Hydrogen assisted biological biogas upgrading

Bassani, Ilaria

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Hydrogen assisted biological biogas upgrading

Ilaria Bassani

PhD Thesis
May 2017

DTU Environment
Department of Environmental Engineering

Hydrogen assisted biological biogas upgrading

Ilaria Bassani

PhD Thesis
May 2017

DTU Environment
Department of Environmental Engineering
Technical University of Denmark

Hydrogen assisted biological biogas upgrading

Ilaria Bassani

PhD Thesis, May 2017

Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljøvej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: reception@env.dtu.dk

Cover: GraphicCo

Preface

This PhD thesis, entitled “Hydrogen assisted biological biogas upgrading”, comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from December 15, 2013 to December 14, 2016. Professor Irini Angelidaki and researcher Panagiotis Kougias were main supervisor and supervisor, respectively.

The thesis is organized in two parts: the first part puts the findings of the PhD into context in an introductive overview; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I Bassani, I., Kougias, P. G., Treu, L., Angelidaki, I. (2015). Biogas upgrading via hydrogenotrophic methanogenesis in two-stage Continuous Stirred Tank Reactors at mesophilic and thermophilic conditions. Environmental science & technology, 49(20), 12585-12593.

II Bassani, I., Kougias, P. G., Angelidaki, I. (2016). In-situ biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresource Technology, 221, 485-491.

III Bassani, I., Kougias, P. G., Treu, L., Porté, H., Campanaro, S., Angelidaki, I. (2017). Optimization of hydrogen dispersion in thermophilic up-flow reactors for ex-situ biogas upgrading. Bioresource Technology, 234, 310–319.

IV Treu, L., Kougias, P. G., Campanaro, S., Bassani, I., Angelidaki, I. (2016). Deeper insight into the structure of the anaerobic digestion microbial community; the biogas microbiome database is expanded with 157 new genomes. Bioresource technology, 216, 260-266.

V Treu, L., Campanaro, S., Kougias, P. G., Sartori, C., Bassani, I., Angelidaki, I. (2017). Genome-centric view of microcosms inhabiting thermophilic and mesophilic biogas upgrading reactors. Manuscript under preparation for submission to Biotechnology for biofuels.

In this online version of the thesis, **paper I-V** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

Acknowledgements

First of all, I wish to thank my supervisor Professor Irini Angelidaki for giving me the opportunity to grow and develop personally and professionally during these three years at DTU.

Likewise, I would like to thank my supervisors, Dr. Panagiotis Kougias and Dr. Laura Treu, for teaching me everything I learned during my time as PhD student and for supporting and helping me all the time with the experiments and the writing.

Special thanks go to Dr. Stefano Campanaro, from University of Padova, for his important contribution to bioinformatics analysis and his constant help with microbiology and molecular biology.

Moreover, I want to thank Hector Diaz and Hector Garcia for the technical and personal support and for the funny time passed in the lab, even when anything seemed to work! ☺

Additionally, I would like to thank Anne Harsting and Charlotte Lind for their kindness and their constant and essential secretarial assistance and Andreas Olesen and Marie Kampmann Eriksen for the Danish translation of the summary.

Also I wish to thank all my colleagues who shared this adventure with me and all my friends for being a necessary distraction, but also a fundamental support during hard times.

At last, I would like to thank my parents who made me who I am and encouraged me every day, and my boyfriend who understood and accompanied me from afar, during this journey!

Summary

Wind and biomass are promoted worldwide as sustainable forms of energy. Anaerobic digestion of biomass produces biogas with ~50−70% CH_4 and 30−50% CO_2. However, biogas with >90% CH_4 content has higher heating value, can be injected into the natural gas grid or used as alternative to natural gas as vehicle fuel. Methods currently available for biogas upgrading mainly consists of physicochemical CO_2 removal, requiring the use of chemical substances and energy input and, thus, increasing process costs.

This PhD project proposes an alternative to existing biogas upgrading technologies, where H_2, produced by water electrolysis, using excess of electricity from wind mills, is coupled with the CO_2 contained in the biogas to convert them to CH_4. This process is defined as biological biogas upgrading and is carried out by hydrogenotrophic methanogenic archaea that couples CO_2 with H_2 to produce biomethane, via hydrogenotrophic methanogenesis. This reaction results in an increment of the total volume of CH_4 produced avoiding any loss of CH_4. Moreover, the CO_2 is converted rather than being released to the atmosphere providing enhanced environmental benefits of biogas technologies. Moreover, hydrogenotrophic methanogenesis can operate in moderate operating conditions, without using chemical substances, and exploiting mixed culture, rather than pure culture, markedly reducing operation costs. The combination of these characteristics makes biomethane an energy carrier with exceptional potential, which could become a key element in the future renewable-based energy system.

Nevertheless, the direct injection of H_2 in the reactor (in-situ biogas upgrading) can cause scientific challenges, such as pH increase due to the CO_2 removal and process inhibition due to higher H_2 partial pressure. Therefore, ex-situ biogas upgrading emerged as a solution aiming at the optimization of the upgrading process in dedicated external reactors. In this concept, biogas and H_2 are introduced into an anaerobic reactor containing a mixed hydrogenotrophic culture where the biogas is upgraded to higher CH_4 content.

To overcome the issues related to in-situ biogas upgrading, a two-stage Continuous Stirred Tank Reactor (CSTR) was designed. In this configuration, the biogas and the digestate produced in the first reactor were transferred to the second one, where H_2 was injected, decoupling biogas production (mainly occurring in the first reactor) and biogas upgrading (occurring in the second reactor) and providing higher process efficiency. Moreover, biogas production and upgrading performances at mesophilic and thermophilic conditions

were compared. The results demonstrate the feasibility of the biogas upgrading process, at both temperature conditions with higher biomethanation and CO_2 conversion efficiency at thermophilic. Moreover, upon H_2 addition, the produced biogas was upgraded to average CH_4 content of 89% in the mesophilic reactor and 85% in the thermophilic.

Nevertheless, H_2 is known to be poorly soluble in aqueous media and its transfer to the reactors' liquid phase represents a strong limiting factor for H_2 availability for methanogens. Therefore, the optimization of H_2 dispersion is crucial to ensure efficient biogas upgrading process. Gas transfer to the liquid phase is specific for given reactor configuration and operating conditions and can be modulated by adjusting on parameters such as mixing speed, gas recirculation and H_2 diffusion device.

This aspect has been investigated in a thermophilic granular up-flow anaerobic sludge blanket (UASB) reactor connected to a separate H_2-injection chamber, for in-situ biogas upgrading. The effect of liquid and gas recirculation on gas-liquid transfer was evaluated. Moreover, the application of different packing materials in the separate chamber, as a mean to minimize gas bubble size and thus increase the gas dissolution in the liquid was discussed. Finally, the effect of gas retention time was evaluated in different chamber configurations to elucidate its role for CO_2 and H_2 conversion to CH_4. It was observed that by distributing H_2 through a stainless steel diffuser followed by a ceramic sponge in a separate chamber (having a volume of 25% of the reactor) and by applying a moderate gas recirculation, CO_2 content in the biogas dropped from 42 to 10% and the final biogas was upgraded from 58 to 82% CH_4 content. Based on these finding, further enhancement of the H_2 gas-liquid mass transfer rate was investigated in four up-flow reactors for ex-situ biogas upgrading. The effect of different H_2 distribution devices and different pore sizes on H_2 uptake by methanogens was elucidated. Moreover the role of input gas flow rate and gas recirculation on H_2 and CO_2 conversion to CH_4 was investigated. The results showed that the configurations involving diffusion devices with larger pore size presented the best kinetics and output-gas quality and at the highest recirculation rate tested, they managed to convert all the input H_2 and CO_2 into CH_4, up to a H_2 loading rate of 3.6 $L/L_{REACTOR}.d$. Accordingly, the CH_4 content in the reactor increased from 23 to 96% and the CH_4 yield reached 0.25 L_{CH4}/L_{H2}.

Finally, to provide higher process control and efficiency, a better understanding of the biogas community composition is crucial. Previous studies have showed that in each microbial community there is a fraction of microorganisms that is always present and constitutes the core of the community and a

fraction depending on operating conditions. Therefore, we hypothesized that the H_2 addition would selectively stimulate the hydrogenotrophic pathway enhancing the CO_2 consumption and thus the biogas upgrading. Based on this knowledge, different bioinformatics approaches, comprising the commonly utilized, 16S rRNA amplicon sequencing, but also Assembled Full-Length 16S rRNA gene sequencing and total random sequencing followed by *de novo* assembly and by a binning strategy, were applied to the study of biogas production and upgrading communities. Specifically, biogas core community was composed of several recurrent microbial groups, including resilient methanogenic archaea such as *Methanoculleus* and *Methanotermobacter* and bacteria belonging to phylum *Proteobacteria* and genus *Synthrophomonas*. Moreover, upon H_2 addition, the concomitant proliferation of hydrogenotrophic methanogens and syntrophic bacteria, such as *Desulfovibrio,* and some *Thermoanaerobacteraceae* and *Syntrophomonadaceae*, and the reduction of aceticlastic methanogens and fermentative bacteria state the role of the H_2 moving biomethanation process toward the final steps stimulating CO_2 consumption and therefore biogas upgrading.

Dansk sammenfatning

Over hele verden bliver vind og biomasse promoveret som bæredygtige energiformer. Ved anaerob udrådning produceres biogas med ca. ~50-70% CH_4 og 30-50% CO_2. Biogas med >90% CH_4 har dog en højere brændværdi og kan derfor indføres i naturgasnettet eller bruges som alternativ til naturgas som brændstof i biler. De metoder der i øjeblikket er tilgængelige til opgradering af biogas er hovedsageligt fysik-kemisk fjernelse af CO_2, hvilket kræver både kemikalier og energi, som forøger procesomkostningerne.

I dette Ph.d.-projekt præsenteres derfor et alternativ til de eksisterende opgraderingsteknologier, hvor H_2 produceret ved elektrolyse af vand ved brug af overskudselektricitet fra vindmøller, blandes med CO_2 i biogassen og omdannes til CH_4. At CO_2 bliver omdannet i stedet for at blive udledt til atmosfæren, øger de miljømæssige fordele ved brug af biogasteknologier yderligere. Derudover kan biologisk metanogenese finde sted ved uden brug af kemiske stoffer, og blandede bakteriekulturer kan bruges i stedet for rene stammer, hvilket kan nedsætte operationsomkostningerne betydeligt. Kombinationen af disse egenskaber gør biometan til en energibærer med enestående potentiale, som kan blive en essentiel del af fremtidens bæredygtige energisystem.

Direkte injektion af H_2 i biogasreaktoren (ved in-situ biogasopgradering) kan dog give tekniske udfordringer, såsom en forøgelse af pH grundet CO_2-fjernelse samt procesinhibering grundet højere H_2-partialtryk. Ex-situ biogasopgradering, hvor den producerede biogas opgraderes i en separat reaktor, kan være en løsning på disse udfordringer. I denne løsning bliver biogas og H_2 introduceret i en separat anaerob reaktor, indeholdende blandede methanproducerende kulturer, hvor biogassen bliver opgraderet til et højere CH_4-indhold.

En to-trins Continuous Stirred Tank Reactor (CSTR) blev designet for at imødegå problemerne ved in-situ biogasopgradering. Her blev biogassen og digestatet, der produceres i den første reaktor, overført til den anden reaktor, hvor H_2 blev injiceret. Dette afkobler biogasproduktionen (der hovedsageligt sker i den første reaktor) fra biogasopgraderingen (som sker i den anden reaktor) hvilket medfører en højere proceseffektivitet. Derudover blev biogasproduktionen og opgraderingseffektiviteten ved termofile og mesofile forhold sammenlignet. Resultaterne viser at det er muligt at udføre biogasopgraderingsprocessen ved begge temperaturforhold men at produktionen af biometan og CO_2-omdannelseseffektiviteten er højere ved termofile forhold. Biogassen blev opgraderet til et CH_4-indhold på 89% i den

mesofile reaktor og et CH_4-indhold på 85% i den termofile reaktor efter tilførsel af H_2.

H_2 er kendt for at have lav opløselighed i vandige medier, så overførsel af H_2 til reaktorens væskefase er derfor en stærkt begrænsende faktor for H_2's tilgængelighed for metanogener. Optimering af H_2-dispersionen er derfor afgørende for at sikre en effektiv biogasopgraderingsproces. Gasoverførsel til væskefasen afhænger af reaktor-konfigurationen og operationsforhold, som igen afhænger af parametre såsom omrøringsintensiteten, gasrecirkulation og metode til H_2-injicering.

Disse parametre blev undersøgt i en termofil granulær Up-flow Anaerobic Sludge Blanket (UASB) reaktor, der var tilsluttet et separat H_2-injektionskammer til in-situ biogasopgradering. Effekten af væske- og gasrecirkulering på gas-væske overførsel blev evalueret. Ydermere blev virkningen af forskellige pakningsmaterialer undersøgt med af indsatte disse i et separat kammer for at minimere gasboblestørrelsen og derved forøge gasopløseligheden i væsken. Afslutningsvis blev effekten af gassens opholdstid for forskellige kammerkonfigurationer evalueret for at belyse dets rolle i forbindelse med CO_2 og H_2's omdannelse til CH_4. Ved at tilføre H_2 gennem et diffusionsapparat af rustfrit stål efterfulgt af en keramisk svamp i et andet kammer (med et volumen på 25% af reaktorens) og sikre and tilpas gasrecirkulation, kunne CO_2-indholdet i biogassen nedsættes fra 42% til 10% og metanindholdet opgraderes fra 58% til 82%. Ex-situ biogasopgradering blev undersøgt i fire up-flow reaktorer, for at forbedre H_2-gas-væske-massefordelingsraten yderligere. Metanogenernes H_2-optag ved forskellige H_2-fordelingsmetoder og forskellige porestørrelser blev undersøgt. Endvidere blev det undersøgt hvilken rolle input gas flowraten og gasrecirkulationen spiller i forbindelse med omdannelse af H_2 og CO_2 til CH_4. Resultaterne viste, at konfigurationerne, med store porestørrelser diffusionsaggregatet gav den bedste kinetik og gaskvalitet. Med den højeste testede recirkulationsrate opnåedes en total omdannelse af H_2 og CO_2 til CH_4, op til en H_2-tilførselsrate på 3.6 $L/L_{REACTOR}$.d. Helt nøjagtigt steg CH_4-indholdet fra 23% til 96% og udbyttet af CH_4 nåede 0.25 L_{CH4}/L_{H2}.

Det er afgørende at opnå en bedre forståelse af den mikrobielle sammensætning i biogasreaktoren for at sikre bedst mulig proceskontrol samt en højere effektivitet. Foregående studier har vist, at der i et hvert mikrobielt samfund vil være en fraktion af mikroorganismerne der altid er til stede og som udgør kærnen af det mikrobielle samfund, mens en anden fraktion af mikroorganismerne afgøres af procesforholdene. Derfor har vi den hypotese, at tilførsel af H_2 specifikt vil stimulere de hydrogenotrofiske archaer og

forøge CO_2-forbruget og derved biogasopgraderingen. På denne baggrund, blev forskellige bioinformatiske metoder anvendt, herunder den udbredte 16S rRNA amplicon sekventering, men også den ”Assembled Full-Length 16S rRNA” gensekventering og ”total random” sekventering efterfulgt af *de novo* samling samt en binning strategi, for at undersøge den mikrobielle sammensætning under biogasopgradering.

Specielt kærnen i biogassens mikrobielle samfund er sammensat af flere af de samme mikrobielle grupper, herunder modstandsdygtige metanogene achaeer såsom *Methanoculleus* og *Methanotermobacter* samt bakterier tilhørende rækken *Proteobacteria* og slægten *Synthrophomonas*. Efter tilførsel af H_2, afgør tilvæksten af hydrogenotrofe metanogener og syntrofiske bakterier (såsom *Desulfovibrio,* og nogle *Thermoanaerobacteraceae* og *Syntrophomonadaceae*) samt reduktionen af acetiklastiske metanogener og fermenterende bakterier H_2’s effekt i forhold til at skubbe produktionsprocessen mod de sidste trin og stimulere CO_2-forbruget og derved biogasopgraderingen.

Table of contents

1 Introduction

1.1 Background

Worldwide the energy demand is increasing and the world's primary source of energy for production of fuels is oil (Cherubini, 2010). Due to the increasing price of fossil fuels, their uncertain availability and their environmental concerns, the possibility for oil exploitation is expected to reduce in the next years (Cherubini, 2010). Therefore, alternative options pointing to limit the impact on climate and decrease the dependence on fossil fuels should be developed.

Denmark is one of the European Countries adopting the most ambitious goals for the transition to renewable energy (RE) system. By 2050, the country aims at being fossil fuel free and at reducing by 50% its total energy use (Turconi et al., 2014). Moreover, Denmark is the European Country with the highest wind electricity penetration in its electric system and the Danish Government has set a goal to cover 50% of electricity demand, by 2020, using wind energy (Hamelin et al., 2011). Additionally, it is a goal to use up to 50% of the manure for bioenergy production.

Nevertheless, such a high electricity penetration will require energy storage, balancing services for the fluctuating supply and demand for electricity and heat, and/or long distance interconnections between regions and countries (Energinet.dk, 2014). Therefore, being foreign storage capacities uncertain in the long run, the development of national energy storage is necessary to ensure system flexibility and security of supply (Energinet.dk, 2015).

1.2 Anaerobic digestion process and microbiology

Anaerobic digestion (AD) is a biological process in which, in absence of oxygen, organic matter is decomposed to biogas and digestate. Biogas produced from AD is mainly composed by CH_4 (~50−70%) and CO_2 (~30−50%). While digestate is a mixture of mineral products (N, P, K, Ca, etc.) resulting from the mineralization of organically bounded nutrients contained in the digested matter (Weiland, 2010). AD process occurs by a combination of pathways assigned to a complex and specialized microbial consortium, where bacteria and archaea coexist maintaining syntrophic relations (Liu and Whitman, 2008). The main steps of the AD process are described below and shown in Figure 1.

1.2.1 Hydrolysis

During AD of organic compounds, biopolymers, such as carbohydrates, proteins and lipids, are hydrolyzed to soluble monomers such as, monosaccharides, amino acids and long chain fatty acids and glycerol, respectively, by action of extracellular enzymes (Ramsay and Pullammanappallil, 2001). Hydrolysis is the rate-limiting step of the AD process (Pavlostathis and Giraldo‐Gomez, 1991) strongly depending on macromolecules composition and structure. For instance, among carbohydrates, cellulose and hemicellulose are quite accessible, while lignin is almost nondegradable (Tong and McCarty, 1991; Yang et al., 2009). Moreover, semisoluble globular proteins are easier degradable compared to fibrous ones (McInerney, 1988). Conversely, lipids degradation is more related to environmental conditions and particle size, rather than molecular composition and structure.

Previous studies showed that, proteolytic bacteria acting during AD process are mainly represented by members of class *Clostridia*, which are also capable for amino acids degradation (Ramsay and Pullammanappallil, 2001). Moreover, both *Clostridia* and phylum *Bacteroidetes* play a fundamental role for carbohydrates degradation, while *Proteobacteria*, such as *Advenella* and *Pseudomonas*, are also involved in lipids metabolism (Ntougias et al., 2013; Xia et al., 2014; Yue et al., 2013).

1.2.2 Acidogenesis

During acidogenesis step, hydrolysis products are converted into volatile fatty acids (VFA), alcohols and H_2. Among them, monosaccharides and amino acids are the most abundant substrates that are rapidly degraded in a wide range of operating conditions (Angelidaki et al., 2011). Examples of microorganisms involved in acidogenesis belong to phylum *Bacteroidetes*, in particular families *Rikenellaceae*, *Bacteroidaceae* and *Porphyromonadaceae* (Hahnke et al., 2015; Traversi et al., 2012; Wang et al., 2014), but also members of the newly characterized phylum *Synergistetes*, such as *Anaerobaculum* and *Aminobacterium* (Stolze et al., 2015).

During an efficient AD process, most of the organic material is directly transformed to methanogenic substrates, such as H_2, CO_2 and acetate, while the remaining portion (~30%) is converted to other VFA and alcohols (Weiland, 2010). However if methanogenic substrates are not efficiently utilized, this portion can increase, with consequent VFA accumulation. For example, dissolved H_2 has a role for products' levels regulation. In fact, high H_2 partial

pressure (P_{H2}) leads to propionate and butyrate accumulation, while low P_{H2} enhances CO_2 and CH_4 production (Liu and Whitman, 2008).

1.2.3 Acetogenesis

During acetogenesis step, products of acidogenesis (mainly propionate and butyrate) are converted to acetate and H_2 by hydrogen-producing acetogens. However, this reaction is limited by the unfavourable energetics of the process and can occur only if intermediates' concentrations are kept low by methanogens or other hydrogen-utilizing bacteria (Angelidaki et al., 2011). This syntrophic relation between hydrogen producers and hydrogen consumers is known as interspecies hydrogen transfer. Alternatively, acetate can be produced from H_2 and CO_2 by homoacetogenic bacteria that are in competition with hydrogenotrophic methanogens for the substrates (Drake, 1994). Resulting acetate can be converted to CH_4 either by direct aceticlastic methanogenesis or through syntrophic acetate oxidation (SAO, i.e. oxidation of acetate to CO_2 and H_2), which is the reversal of the homoacetogenic pathway (Wood–Ljungdahl pathway (WLP))(Angelidaki et al., 2011). SAO can be performed by different groups of microorganisms, such as *Clostridium ultunense* and *Thermotoga lettingae* and families *Syntrophomonadaceae* and *Thermoanaerobacteriaceae* (Hattori, 2008).

1.2.4 Methanogenesis

Methanogenesis is the final step of AD process where mainly acetate, H_2 and CO_2 are converted to CH_4 by methanogenens.

Methanogens belong to the *Archaea* domain, phylum *Euryarchaeota*. Sixth phylogenetic orders of methanogens have been identified: *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, *Methanopyrales* and the recently identified *Methanocellales* (Garcia et al., 2000; Sakai et al., 2008).

Most of the CH_4 is formed from acetate, by aceticlastic methanogens belonging to order *Methanosarcinales* (*Methanosarcina* spp. and *Methanosaeta* spp.) or through SAO, and only a minor part is produced directly from H_2 and CO_2 (Liu and Whitman, 2008; Schnürer et al., 1999). This reaction is carried out by hydrogenotrophic methanogens that reduce CO_2 with H_2 to CH_4.

Among *Methanosarcinales*, *Methanosarcina* spp. can perform methanogenesis from both acetate and H_2/CO_2, while *Methanosaeta* spp. is strictly aceticlastic. Conversely, the other orders of methanogens mentioned produce CH_4 mainly from H_2/CO_2 and are not able to utilize acetate as substrate for methanogenesis.

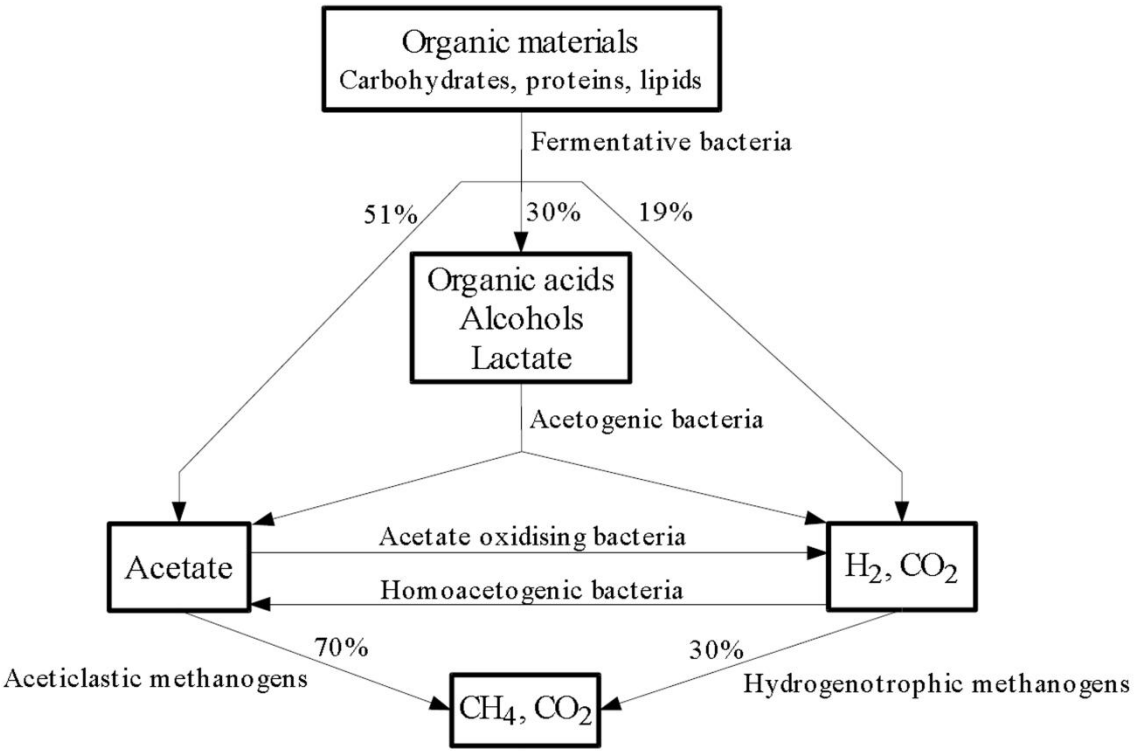


Figure 1: Schematic description of the AD process (adapted from Angelidaki and Ellegaard (2002)).

1.3 Biogas upgrading

Biogas' energy content is determined by the heating value of its methane fraction. Therefore, the ~40% of CO_2 contained in the biogas dilutes almost by half its energy density limiting the use of biogas to heat or combined heat and power (CHP) generation.

In order to overcome these limitations, biogas can be upgraded to natural gas quality, i.e. to biomethane. This involves the removal of CO_2 and water vapour, as well as typical contaminants, such as sulphur gases, siloxanes, dust and particles. Upgraded gas can be injected into the natural gas grid, compressed and transported, or used as alternative to natural gas as vehicle fuel (Deng and Hägg, 2010). In addition, biomethane can be converted back to electricity transforming the natural gas grid into a vast energy storage system (Thrän et al., 2014).

Therefore, biogas upgrading presents several advantages, such as territorial and temporal decoupling of energy generation and use, and possibility for large-scale storage and transport. The combination of these characteristics makes biomethane an energy carrier with exceptional potential, which could become a key element in the future renewable-based energy system.

Available, biogas upgrading technologies are described below.

1.3.1 Conventional biogas upgrading

Currently, there are five commercially available technologies for biogas upgrading and another one is in developing phase. A brief overview is reported below according to Sun et al. (2015), Thrän et al. (2014) and Petersson and Wellinger (2009).

- **Water physical scrubbing:**

During this process, the biogas is pressurized (5–10 bar) and introduced from the bottom of an absorption column, where the CO_2 is dissolved in water. The water is then circulated into a flush column at lower pressure (2.5–3.5 bar). This enables the separation of most of the CH_4 and some of the CO_2 dissolved in the water. Successively, the water enters from the top of a desorption column, while air is introduced from the bottom at atmospheric pressure, allowing the CO_2 to be released from the water (air stripping). Both absorption and desorption columns are filled with packing material in order to maximize the contact surface between the water and the gasses.

- **Organic solvent physical scrubbing:**

This technology uses the same principle as water scrubbing, except for the use of organic solvents instead of water, due to the higher solubility of CO_2 in organics solvent compared to water. Compared to water scrubbing, this method presents the advantage that lower liquid flows are required allowing the use of smaller column's diameters. Conversely, cooling and heating of the solvent are needed during the procedure.

- **Amine chemical scrubbing:**

This technology uses a water solution of amines that chemically binds with CO_2 molecules in the biogas. The most common amines currently utilized are a mixture of monoethanolamine (MEA) and piperazine (PZ), usually named activated MDEA (aMDEA). The amine scrubbing consists of an absorber, where CO_2 (and H_2S) is removed from the biogas, and a stripper, which regenerates the amine solution by releasing the CO_2 into the atmosphere. Both modules require pressure application and are filled with packing material to maximize gas-liquid contact surfaces. Various heat exchangers are implemented in the system to minimize heating and cooling demands.

- **Pressure swing adsorption (PSA):**

This procedure utilizes an adsorbent material to separate the gasses according to their physical properties. The materials used are porous and with high spe-

cific area. Examples are activated carbon, zeolites, silica gels and carbon molecular sieves, which are able to selectively absorb the CO_2 from the biogas. A drawback of this technology is that ~4% CH_4 is lost within the off-gas stream.

- **Membranes:**

Membrane upgrading technology is based on the use of filters able to separate the different biogas components according to their molecular size. Selected membrane materials, such as polymeric hollow fibres or carbon membranes, retain most of the CH_4, while most of the CO_2, together with water vapour, and H_2S permeates through the membrane. The driving force of the process is the pressurisation of the biogas to 6–20 bar.

- **Cryogenic separation:**

Cryogenic technologies for biogas upgrading are at developing stage and aim to separate the gases contained in the biogas according to their boiling temperature, by gradually cooling the gas. Several cooling techniques are investigated testing different combinations of compressors, heat exchangers and expansion devices.

Because of the working conditions required for the operation of these upgrading technologies, investment and operational costs due to energy demand remain high (Sun et al., 2015). Energy demand is mainly derived by the electricity consumption and by the use of water or chemicals and heating/cooling devices (Sun et al., 2015). Moreover, the use of organic solvents, the high amount of CO_2 released to the atmosphere and the CH_4 losses reduce the environmental benefits of biogas production.

1.3.2 Biological biogas upgrading

Biological biogas upgrading proposes an alternative to the currently available technologies, where the CO_2 contained in the biogas is coupled with external H_2 and converted to additional CH_4, via hydrogenotrophic methanogenesis (Figure 2).

Hydrogenotrophic methanogens use H_2 as reducing agent to convert CO_2 to CH_4 according to equation 1. This reaction induces an electrochemical gradient across their cell membrane allowing the formation of ATP through a process named chemiosmosis. This constitutes their energy source. Additionally, some of the H_2 and CO_2 are used as elemental sources for cell growth (Bryant, 1979).

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \quad \text{(Eq. 1)}$$

This reaction results in an increment of the total volume of CH_4 produced avoiding any loss of CH_4. Moreover, the CO_2 is converted rather than removed and it is not released to the atmosphere providing enhanced environmental benefits of biogas technologies. Moreover, hydrogenotrophic methanogenesis can operate exploiting mixed culture, rather than pure culture, and can be applied in mild operating conditions, without using chemical substances, markedly reducing operation costs (Götz et al., 2015).
Biological biogas upgrading can be achieved by injecting external H_2 directly in the anaerobic reactor (in-situ biogas upgrading) (Bassani et al., 2016). Nevertheless, the direct H_2 injection in the reactor can cause technical challenges. Primarily, CO_2 removal could lead to a substantial increase of pH negatively affecting the process (Luo et al., 2012). Moreover, as described in section 1.2.2, the increase of P_{H2} can cause VFA accumulation inhibiting the process (Luo et al., 2012). Hence, ex-situ biogas upgrading emerged as a solution aiming at the optimization of the upgrading process in dedicated external reactors. In this concept, biogas and H_2 are introduced into an anaerobic reactor containing a mixed hydrogenotrophic culture and the biogas is upgraded to higher CH_4 content (Kougias et al., 2016a). However, because H_2 is 500 times less soluble in water than CO_2 (Ahern et al., 2015), the H_2 transfer to the liquid phase, to make it available for microorganisms, remains the limiting factor of both in-situ and ex-situ processes (Díaz et al., 2015; Luo and Angelidaki, 2013a). Therefore, it is crucial to optimize this technology to overcome H_2 gas-liquid transfer limitation. Gas transfer coefficient($k_L a$) is specific for given reactor configuration and operating conditions (Pauss et al., 1990). Thus, it can be modulated by changing parameters such as mixing speed (Kramer and Bailey, 1991; Luo and Angelidaki, 2012), gas recirculation (Guiot et al., 2011) and H_2 diffusion device (Díaz et al., 2015; Luo and Angelidaki, 2013b) to reduce H_2 bubbles' size and maximize the gas-liquid contact.

Besides, in both in-situ and ex-situ concepts, utilized H_2 also derives from RE sources. For instance, H_2 gas can be produced from biomass gasification, reforming of biomethane, biological H_2 production, or through electrolysis of water (Turner et al., 2008).
The best option in the context of this project is to produce H_2 with electrolyzes powered by off-peak electricity surplus from wind and/or solar power (Figure 2). In fact, in northern EU countries, such as Denmark, >26% of the electricity produced from wind mills is a temporary surplus. Therefore, the ability to use surplus electricity may improve the overall efficiency of the

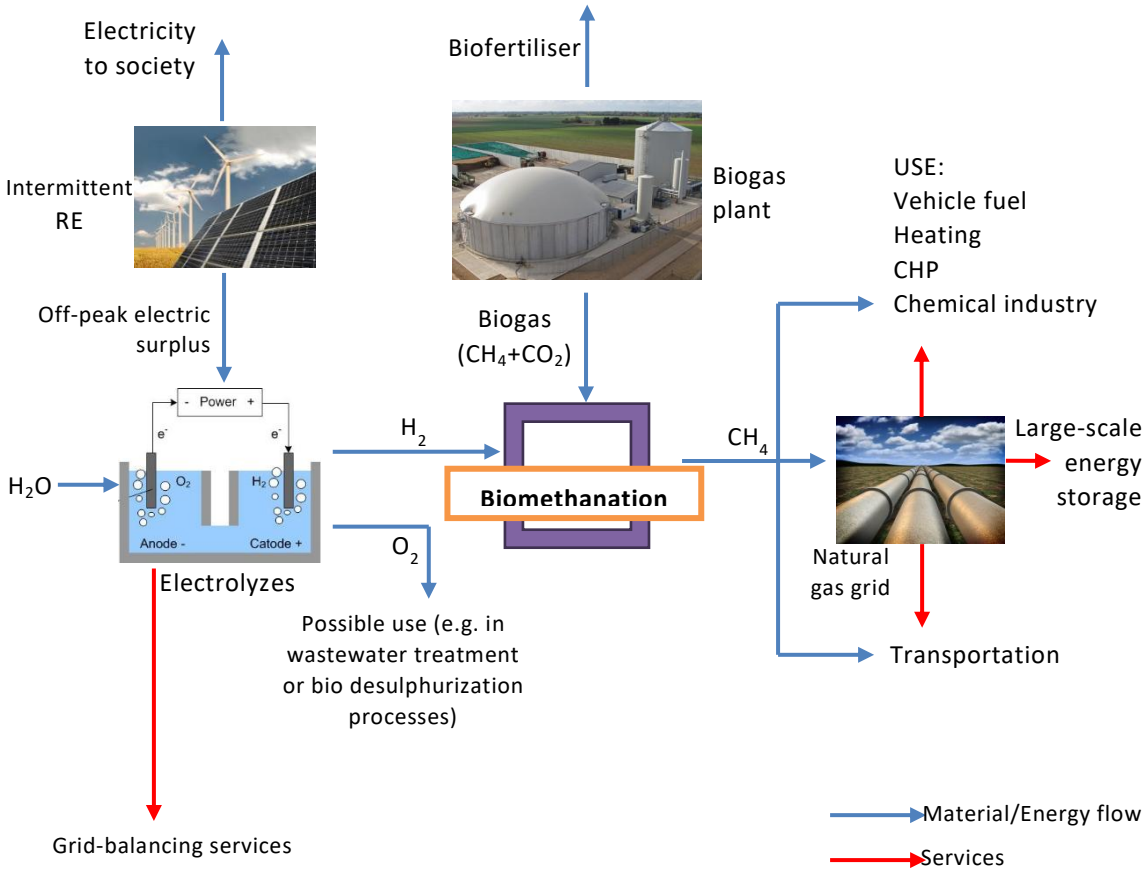


Figure 2: Energy flowchart in biological biogas upgrading.

power system (Carton and Olabi, 2010; Sharman, 2005; Sovacool, 2013). Electrolysis of water consists of breaking water into H_2 and O_2 with direct electric current passing through two electrodes and a membrane, according to equation 2

$$2\,H_2O\,(l) = 2\,H_2(g) + O_2\,(g) \qquad \text{(Eq. 2)}$$

The reaction takes place in an electrochemical cell, containing two electrodes (anode and cathode, interconnected through an external circuit), an electrolyte (a substance that increases the electrical conductivity between the electrodes) and a membrane that prevents the produced gases from recombining back into water. The reduction occurs at the cathode, while the oxidation takes place at the anode. Ions act as charge carriers and are transported through the membrane to close the circuit between the two electrodes (Benjaminsson et al., 2013). Within this concept, the excess electricity could be directed to the electrolyser to produce and stores H_2. When the turbine shuts off due to low wind speed the H_2 would be channelled to the electricity generator to fulfil the electricity demand, while when the demand is low and the wind speed high the turbine moves the electricity again to the electrolyser

(Carton and Olabi, 2010). Because H_2 itself is an energy carrier that can be converted into electricity, theoretically it could be store and use for electricity generation or as vehicle fuel. However, H_2 has low density and small molecular size resulting in storage limitations and high costs related to technical complications (Holladay et al., 2009; Jürgensen et al., 2014). Therefore, the use of H_2 derived from off-peak electric surplus from RE sources for biogas upgrading can overcome the limitations of H_2 management and, at the same time, can lower the costs associated with biogas upgrading technologies (Götz et al., 2015; Jürgensen et al., 2014).
Moreover, from a biological viewpoint, intermittent H_2 feed of methanogens has been proven feasible, showing that mixed hydrogenotrophic culture can rapidly restart CO_2 and H_2 conversion (Martin et al., 2013).

1.4 Metagenomic approaches for biogas microbial community characterization

As previously described, the AD process comprises a series of complex reactions driven by different microbial groups. Nevertheless, the most of the microbial consortium involved in this process in currently unknown (Liu and Whitman, 2008). Therefore, a better understanding of AD community composition is crucial for process control and optimization. Unfortunately, only a small fraction of the biogas microbiome is represented by cultivated organisms. This bottleneck can be overcome using metagenomics, in which genomic DNA is extracted from environmental samples and directly sequenced (Albertsen et al., 2013). Metagenomics can be defined as the direct genetic study of environmental samples without microorganisms' cultivation and provides information about microorganisms' phylogeny and abundance (Hess et al., 2011; Iverson et al., 2012).
During the last decades, next generation sequencing (NGS) technology has been exceptionally developed giving an essential contribution to several research fields, such as metagenomics. This technology can generate up to 4 billion reads in a single run, compared to one single sequence at the time achievable by Sanger sequencing. Three commercial platforms are currently available. They are named Roche 454 Genome sequencer, Illumina Genome Analyzer and Life Technologies SOLiD system, and are based on a parallel sequencing process that produce thousands of sequences at the time (Hess et al., 2011; Iverson et al., 2012).

The 16S rRNA gene sequencing is currently the most largely used approach for microbial community taxonomic assignment. This is due to the presence, in its sequence, of highly conserved regions, utilized for primer design, and variable regions, used for microbial identification (Miller et al., 2011). Nevertheless, short read lengths and high conservation of sequences can produce ambiguous assignments (Miller et al., 2011). Therefore, the full-length 16S rRNA gene can be reconstructed providing more complete taxonomic information compared to amplified short hypervariable regions, including undiscovered taxa (Miller et al., 2011). However, due to the complexity of the AD community the most of biogas reactor's population remains uncharacterized. In this context, total random sequencing (TRS) strategy emerged as a solution to achieve the phylogenetic resolution that could not be reached through single-gene approaches (Bassani et al., 2015). Furthermore, previous works demonstrated that the assembly of TRS can markedly improve the reliability of taxonomic annotation (Campanaro et al., 2016; Treu et al., 2016b). Moreover, the application of a binning strategy, a process where scaffolds obtained from *de novo* assembly are clustered in the same individual genome, according to their occurrence, permitted to acquire a deeper insight into the biogas microbial community recognizing a group of common microorganisms that could represent the core of biogas microbial community (Campanaro et al., 2016; Kougias et al., 2016b; Treu et al., 2016b).

1.5 Objectives and thesis structure

1.5.1 General objectives

This PhD project proposes an innovative process in which H_2 produced by water electrolysis using peak load/excess electricity from wind mills, is biologically converted by binding CO_2 to CH_4. This technology has been optimized to provide efficient in-situ and ex-situ biogas upgrading processes. Within the first objective, external H_2 has been combined with the CO_2 in the biogas for biogas production and upgrading. Within the second objective, external H_2 and biogas have been injected to an anaerobic reactor containing a mixed hydrogenotrophic culture for CO_2 conversion to CH_4. Through these processes, biogas upgrading was achieved, giving synergistic advantages for both the overall RE system, with high share of wind power, and for the biogas plants themselves. Moreover, biological carbon fixation performed during ex-situ process is an interesting solution to reduce greenhouse gases emissions.

Starting from the hypothesis that the H_2 addition would selectively stimulate the hydrogenotrophic pathway enhancing the CO_2 consumption and consequently the

biogas upgrading, the effects of H_2 on process' performances and microbiology has been investigated. Moreover, technical solutions to overcome the major limitation of this technology, i.e. H_2 gas-liquid transfer, have been studied in order to enhance H_2 utilization and CO_2 conversion to CH_4.
In this context, different bioinformatics approaches have been applied to analyze biogas upgrading reactor community and achieve a deeper insight into AD microbial population.

1.5.2 Specific objectives and thesis structure

In Chapter 2, main scientific challenges related to in-situ biogas upgrading process, such as reactor's pH and VFA levels increase, have been overcome in two-stage Continuous Stirred Tank Reactors (CSTRs). In this configuration, the biogas and the digestate produced in the first reactor were transferred to the second one, where H_2 was injected, decoupling biogas production (mainly occurring in the first reactor) and biogas upgrading (occurring in the second reactor) and providing high process efficiency. Moreover, biogas production and upgrading performances at mesophilic and thermophilic conditions were compared (Paper I).

The major technical challenge discussed in Chapter 3 was the H_2 low gas-liquid mass transfer rate. In this chapter in-situ biogas upgrading process was investigated in a thermophilic granular up-flow anaerobic sludge blanket (UASB) reactor connected to a separate H_2-injection chamber. The effect of liquid and gas recirculation on gas-liquid transfer was evaluated. Moreover, the application of packing materials in the separate chamber, such as rashig rings and alumina ceramic sponge, as a mean to minimize gas bubble size and thus increase the gas dissolution in the liquid was discussed. Finally, the effect of gas retention time (GRT) was evaluated in single or serial chamber configurations with different working volumes to elucidate their role for CO_2 and H_2 conversion to CH_4 (Paper II).

In Chapter 4, according to the findings reported in the previous chapter, further enhancement of the H_2 gas-liquid mass transfer rate was discussed. This parameter was tested in four up-flow reactors for ex-situ biogas upgrading. The effect of different H_2 distribution devices (metallic diffusers, combined with alumina ceramic sponge, or alumina ceramic membrane) having different pore sizes, on H_2 uptake by methanogens was investigated. Moreover the role of input gas flow rate (Q_{IN}) and gas recirculation (Q_{RC}) on H_2 and CO_2 conversion to CH_4 was evaluated (Paper III).

In Chapter 5, an overview of main bioinformatics approaches utilized in this PhD project to investigate biogas and biogas upgrading microbial communities was provided. In particular, 16S rRNA amplicon sequencing (16S AS), 16S rRNA Shotgun Reads (16S SR), Assembled Full-Length 16S rRNA gene sequencing (16S AFL), TRS and *de novo* assembly and binning strategies were described and the contribution of each approach to the unveiling of reactor's microbial community was explained. The presented bioinformatics approaches are reported in Papers I, III, IV and V.

Chapter 6 described the main findings related to biogas production and upgrading microbial communities acquired during this PhD study. Specifically, an overview of bacterial and archaeal consortia involved in these processes and depicted by applied bioinformatics strategies was provided. Moreover, the effect of H_2 addition on the community composition was elucidated. Finally, the core of biogas microbiome identified through *de novo* assembly and binning strategy was described. Presented results are reported in Papers I, III, IV and V. Conclusions and future perspectives follow.

2 Solving in-situ biogas upgrading challenges in two-stage CSTRs

2.1 Main scientific challenges related to in-situ biogas upgrading

Although biological biogas upgrading presents several advantages, the direct addition of H_2 to the anaerobic reactor (in-situ upgrading) can cause some scientific challenges.

Firstly, the bicarbonate removal from the liquid phase, due to the conversion of CO_2 to CH_4, could results in a considerable increase of pH possibly affecting AD process (Luo et al., 2012). In fact, it is known that methanogenesis takes place within a narrow pH interval (~6.5-8.5) and the process can be seriously inhibited if reactor's pH is not maintained within this range (Weiland, 2010).

Secondly, the conversion of VFA and alcohols to acetate, CO_2, and H_2 is only favourable at P_{H2} <100 Pa (Liu and Whitman, 2008). P_{H2} in anaerobic reactors ranges from 2 to 1200 Pa and when methanogenesis is efficiently performed, H_2 is rapidly metabolized and its concentration is maintained <10 Pa. Conversely, H_2 levels >10 Pa can reduce process efficiency, with consequent accumulation of lactate, ethanol, propionate, and butyrate (Liu and Whitman, 2008). Therefore, during in-situ biogas upgrading, the addition of external H_2 to the anaerobic reactor can increase P_{H2} causing VFA accumulation and process inhibition (Luo et al., 2012).

2.2 An innovative reactor configuration to overcome in-situ biogas upgrading technical issues

In order to overcome the technical issues associated with in-situ biogas upgrading technology, in Paper I, an innovative reactor setup has been proposed (Figure 3). The designed reactor was composed of two CSTRs connected in series, where biogas and digestate produced in the first reactor were transferred to the second one, where H_2 was injected. The configuration was operated at both mesophilic and thermophilic conditions.

The experiment aimed to decouple biogas production, mainly occurring in the primary reactors, and biogas upgrading, taking place in the secondary reactors. Thus, we hypothesized that pH and VFA increase, consequent to H_2 ad-

dition, would occur only in the secondary reactors, without affecting the AD process taking place mainly in primary reactors.

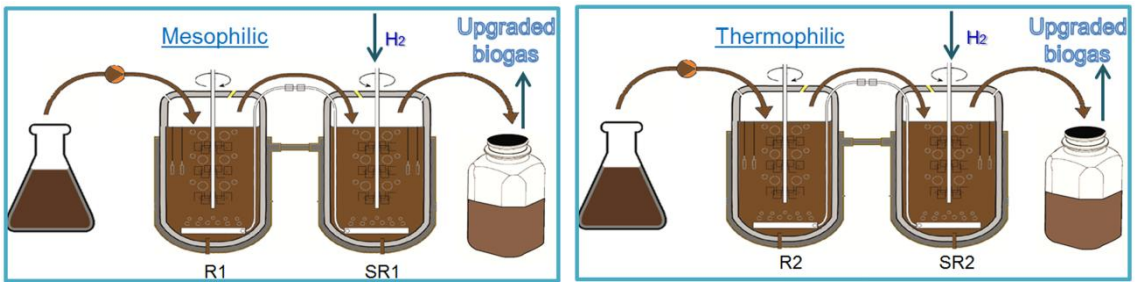


Figure 3: Two stages mesophilic and thermophilic reactor configuration (adapted from Paper I).

The setup consisted of two analogous two-stage CSTRs, each with a total working volume of 3.5 L (1.5 and 2 L for primary and secondary reactor, respectively). Primary reactors were fed with cattle manure, while the secondary were fed with the effluent from the primary. Additionally, the biogas produced in the primary reactors was transferred to the secondary ones. Once steady state conditions were achieved, H_2 was continuously injected to the secondary reactors through a diffuser placed at the bottom of the reactor.

The volumetric H_2 flow rate ($Q_{H_2,IN}$) was defined according to the stoichiometry of hydrogenotrophic methanogenesis reaction and set to 4 times the CO_2 production rate (P_{CO_2}) recorded in the output gas before the H_2 addition (Luo and Angelidaki, 2013b).

2.3 Effect of H_2 addition on two-stage CSTRs at mesophilic and thermophilic conditions

Detailed operational data from the reactors before and after the H_2 addition are reported in Paper I (Table 1 and Figures S1 and S2).

Outcomes of this study showed that, interestingly, most of the biogas production derived from primary reactors (97% and 74% of the total biogas produced, in mesophilic and thermophilic reactor, respectively). Additionally, upon H_2 addition, notably, the portion of CH_4 derived from the conversion of CO_2 to CH_4 represented ~25% of the total CH_4 yield(Y_{CH_4}), in both reactors.

The results indicate that the biogas obtained fulfils the requirements for the injection to the national gas grid or for its use as alternative to natural gas, as fuel for cars (Deng and Hägg, 2010). Indeed, upon the H_2 addition, the CH_4 production rate (P_{CH_4}) increased by 53% and by 45%, in mesophilic and thermophilic reactor respectively, resulting in an average CH_4 content of

~89% (with a maximum of 92%) and of ~85% (with a maximum of 91%), respectively. Therefore, the rate of CO_2 detected in the output gas decreased by 65% and by 77% resulting in an average CO_2 content of 9% (with a minimum of 5%) and 7% (with a minimum of 6%), respectively.

Notably, in both reactors, the decrease of rate of CO_2 detected in the output gas resulted higher than the increase of P_{CH_4}. This can be explained by the concomitant pH increase resulting in a larger portion of CO_2 dissolved in the reactor liquid phase, as bicarbonate. In fact, while in primary reactors pH levels remained stable for the whole experiment, in the secondary, the pH increased up to 8.5. Nevertheless, despite the high pH levels detected, no biomethanation inhibition was observed. This finding was confirmed by a batch assay aiming on determining the hydrogenotrophic methanogenic activity of secondary reactors at different pH levels. According to this test, biomethanation was feasible at a maximum pH of 8.5 (although with marked decreased Y_{CH_4}) stating the adaptation of microorganisms to the increased pH values. Conversely, pH levels above 8.5 resulted in severe process inhibition. Detailed results are reported in Paper I (Figure 1).

Moreover, despite, the higher P_{H2}, consequent to the H_2 addition, could likely result in VFA accumulation (Liu and Whitman, 2008), in the present experiment, VFA levels remained stable and low for the whole experiment, in both primary and secondary reactors Paper I (Figure S4).

From the comparison of biogas production and upgrading performances at mesophilic and thermophilic conditions it was shown that, in accordance with previous studies (Levén et al., 2007), more efficient organic matter degradation (20% more) and higher Y_{CH_4} (55% higher) were detected at thermophilic conditions. Nevertheless, upon H_2 addition, at both operating temperatures, marked improvement of P_{CH_4} and biogas quality and efficient H_2 consumption rates were achieved. However, more efficient CO_2 conversion to CH_4 was observed at thermophilic conditions, compared to mesophilic. Specifically, in the mesophilic reactor, 69% of the produced CO_2 (with a maximum of 87%) was converted to CH_4, compared to the 77% (with a maximum of 84%) converted by the thermophilic. These results demonstrate the superiority of biomethanation process conducted at thermophilic conditions.

3 Improvement of hydrogen dispersion in thermophilic UASB reactor for in-situ biogas upgrading

3.1 Gas–liquid mass transfer as rate limiting factor for efficient H_2 utilization

During AD process, in which different gases are produced, liquid-to-gas transfer plays a fundamental role. While, in typical anaerobic digester operating conditions, the mass transfer of highly soluble gases is not limited, this parameter acquires more importance for poorly soluble gases, such as H_2 (Pauss et al., 1990). In fact H_2, being an intermediate metabolite of the AD process, must be present at low concentrations to allow the occurrence of the methanogenesis step (Liu and Whitman, 2008). Therefore, a better understanding of H_2 transfer to liquid is critical to ensure proper process operation.
Moreover, in H_2 assisted biogas upgrading, H_2 mass transfer rate is a key element, because H_2 must be dissolved in reactor's liquid phase to be utilized by microorganisms. Therefore, this parameter results crucial for determining the available substrate for methanogens (Bassani et al., 2016).
H_2 gas-liquid mass transfer rate can be described by the following equation (3):

$$r_t = 22.4 k_L a (H_{2gTh} - H_{2l}) \quad \text{(Eq. 3)}$$

where r_t (L/(L_R.d) expressed as per litter reactor (L_R)) is the H_2 gas–liquid mass transfer rate, 22.4 (L/mol) is the gas volume to mole ratio (1 mol gas corresponds to 22.4 L at STP), $k_L a$ (day^{-1}) is the gas transfer coefficient, H_{2gTh} (mol/L) is the H_2 concentration in the gas phase, while H_{2l} (mol/L) the H_2 dissolved in the liquid phase. One way to increase r_t is by increasing $k_L a$. This coefficient is determined and strictly correlated with reactor configuration and operating conditions (Pauss et al., 1990). Therefore, $k_L a$ can be modulated by changing parameters such as mixing speed (Bhattacharyya and Singh, 2010; Luo and Angelidaki, 2012), gas recirculation flow (Guiot et al., 2011) and diffusion device (Luo and Angelidaki, 2013a, 2013b).
Based on these concepts a novel reactor configuration was designed to improve H_2 transfer to the liquid phase.

3.2 A novel thermophilic granular UASB reactor for in-situ biogas upgrading

UASB reactors are commonly utilized in industrial wastewater treatment plants (Gomec, 2010; Sevilla-Espinosa et al., 2010). This technology is based on the presence of granular sludge, where microorganisms coexist in aggregates, known as granules. In particular, starch-grown granules are known to contained high abundance of carbohydrate degrading bacteria and hydrogenotrophic methanogens, likely due to their interspecies H_2 transfer with syntrophic bacteria (Lu et al., 2015). Moreover, according to previous studies, H_2 is expected to enhance the hydrogenotrophic methanogenic pathway and the syntrophic relationship between hydrogenotrophic methanogens and bacteria, further increasing their relative abundance in the reactor's granular sludge (Bassani et al., 2015). A suitable substrate for such AD application is potato-starch wastewater produced as by-product from the potato-starch processing industry. In fact, this substrate is known to contain high concentration of biodegradable compounds, such as starch and proteins, suitable for biogas production (Barampouti et al., 2005). Moreover, for in-situ biogas upgrading process, the acidic pH of potato starch wastewater (~6) results beneficial to counteract the pH increase, upon H_2 addition, due to the CO_2 removal. In Paper II, an innovative setup composed of a 1.4 L thermophilic UASB reactor connected to a separate chamber, having 0.2 L volume, where the H_2 was injected, was designed to enhance the H_2 dispersion in the reactor liquid phase for in-situ biogas upgrading (Figure 4). Two identical reactor configurations were operated. One setup was used as upgrading reactor, while the other was utilized as control reactor operated through the experiment without H_2 injection. Once steady state conditions were achieved, H_2 was continuously introduced in the upgrading reactor through a metallic diffuser located in the separate chamber. Liquid recirculation flow rate, known to play an important role in wastewater mixing, improving the contact among granules (Powar et al., 2013; Zheng et al., 2012), has been modulated. Successively, in order to increase H_2 $k_L a$ and, thus, improve H_2 gas-liquid contact, key factors affecting H_2 gas-liquid mass transfer rate have been investigated. For this aim, different gas recirculation flow rates (Q_{RC}) have been tested. Moreover, in order to reduce gas bubble size and thus increase contact surface area between gas and liquid phases the, different packing materials (rashig rings and alumina ceramic sponge) have been introduced to the separate chamber. Finally, the effect of gas retention time (GRT) has been evaluated in single or serial chamber configurations

having different working volumes. The detailed experimental plan has been described in Table 1.

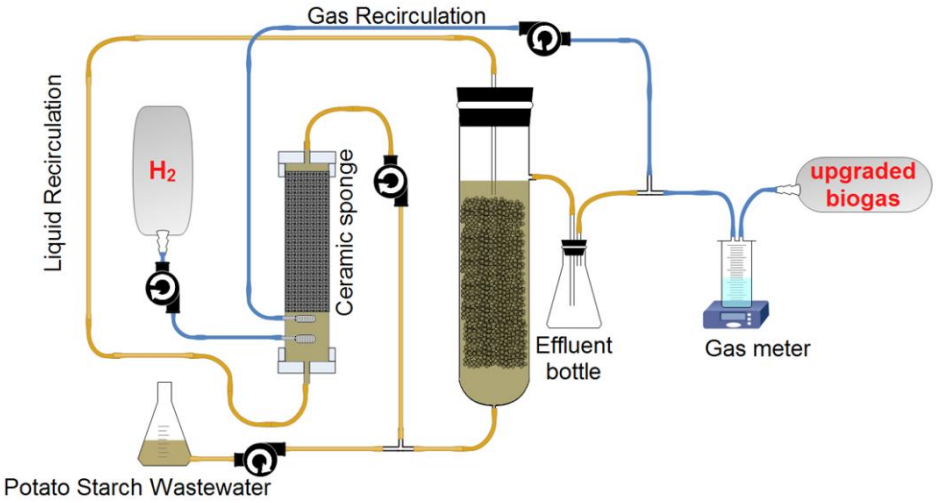


Figure 4: UASB reactor configuration (adapted from Paper II).

Table 1: In-situ biogas upgrading experimental plan.

Phase	Period	H_2 distribution device	Liquid recirculation flow (L/h)	Q_{RC} (mL/min)	$Q_{H_2,IN}$ ($L/L_R.d$)
Pre H_2	I	-	4	-	-
In-situ	II	rashig rings	4	-	3.5
	III	rashig rings	7	-	2.6
	IV	ceramic sponge	7	-	2.6
	V	ceramic sponge	7	4	2
	VI	ceramic sponge	7	6	1.8
	VII	serial chambers	7	6	1.8
	VIII	single chamber with extended length	7	6	1.8

3.3 H_2 dispersion and biogas upgrading in a thermophilic granular UASB reactor

Detailed operational data from the upgrading and control reactors are reported in Paper II (Tables 1 and 2 and Figures 1 and 3).

Based on achieved results, from the application of H_2 to the separate chamber of the upgrading reactor, containing rashing rings (Period II), an increase of 45% CH_4 production rate (P_{CH_4}), compared to the control reactor, was observed. Notably, this improvement of P_{CH_4} was accompanied by the increase

of pH from 7.5 to 7.9. Nevertheless, because of the low H_2 gas-liquid mass transfer rate, only 51% of the H_2 injected was utilized leading to a high amount of unutilized H_2 in the output gas (45%). Additionally, as described in section 2.1, due to the high P_{H2} resulting from the continuous addition of H_2, VFA levels in the upgrading reactor increased from 1 to 3.4 g/L. Moreover, the accumulation of acetate, over other VFA, indicates that AD was shifted towards homoacetogenic pathway, rather than methanogenesis (Cord-Ruwisch et al., 1997). To reduce P_{H2} and increase H_2 utilization, H_2 gas flow rate($Q_{H_2,IN}$) was gradually reduced compared to the amount required according to the stoichiometry of the hydrogenotrophic methanogenesis.

Nevertheless, increased liquid recirculation flow rate, known to provide beneficial effect for both substrates availability for microorganisms (Bhattacharyya and Singh, 2010; Luo and Angelidaki, 2012) and gas-liquid contact (Kramer and Bailey, 1991), did not result in markedly improved upgrading performances (Period III). This result can be explained by the lower concentration of bicarbonate in reactor liquid phase, which probably affected liquid density, reducing granular bed expansion and avoiding the positive effect of the higher mixing provided (Ohsumi et al., 1992; Song et al., 2005).

Conversely, the application of different packing materials in the H_2 injection chamber, such as alumina ceramic sponge, having surface area 160 times higher than rashig rings and allowing H_2 distribution through smaller bubbles, led to improved H_2 utilization efficiency and P_{CH_4} (Period IV). Specifically, 67% of the H_2 injected was utilized reducing the H_2 content in the output gas to 31% and increasing the CH_4 content from 45 to 52%. These results clearly demonstrate the fundamental role of distribution device's porosity and pore size and consequent H_2 bubbles size to ensure an optimal H_2 availability for methanogens.

Additionally, based on the recognized positive effect of gas recirculation on $k_L a$ coefficient (Guiot et al., 2011), different Q_{RC} have been applied to the upgrading reactor (4 and 6 mL/min in Periods V and VI, respectively). The longer GRT and, consequently, the higher H_2 dissolution provided by the gas recirculation led to a more efficient H_2 utilization (87%). Therefore, higher P_{CH_4} derived from the hydrogenotrophic CO_2 conversion were achieved resulting in the increase of CH_4 content in the biogas from 57 to 66% and the decrease of unutilized H_2 from 20 to 14%. As expected, a concomitant increase of pH to 8.2, due to the CO_2 removal, was observed. Despite the positive effect of gas recirculation on upgrading performances, the consequent high pressure generated through the diffuser resulted in turbulent movements

causing granules disintegration and, thus, reduction of reactor's active biomass. This phenomenon can explain the concomitant decrease of P_{CH_4}, derived from the degradation of the liquid substrate, and the increase of VFA levels to >5 g/L observed in the upgrading reactor from period V. These negative effects can be overcome using high porosity diffusers that allow the application of equivalent or higher flows, avoiding excessive pressure and, therefore, turbulent movements.

Successively, to further increase the contact area between H_2 bubbles and liquid the ceramic sponge surface area was doubled, by doubling H_2-injection chamber volume, either by connecting two chambers in series (Period VII), or by assembling them in a single longer chamber (Period VIII). The connection of two chambers in series did not result in substantial improvement of upgrading performances showing that chamber's volume itself has not a direct correlation with H_2 dispersion. Conversely, by assembling two chambers in a single longer one, 94% of the H_2 injected was utilized, resulting in only 8% H_2 remained unutilized in the output gas. Therefore, CO_2 and CH_4 contents in the biogas dropped to 10% and increased to 81% (with a maximum of 82%) respectively. However, due to higher CO_2 converted, the pH raised to 8.4. The results clearly demonstrate the importance of GRT, gas-liquid contact area and, at the same time, of the specific reactor configuration to optimize H_2 dispersion and, thus, CO_2 conversion to CH_4.

4 Enhancement of hydrogen mass transfer rate in ex-situ biogas upgrading up-flow reactors

4.1 Introduction to ex-situ biogas upgrading technology

Climate change is one of the greatest environmental issues of this time and the increase of greenhouse gases emissions, such as CO_2, is strictly connected with this phenomenon. Therefore, there is an urgent need for reducing CO_2 accumulation in the atmosphere. For this aim, several technologies have been developed for CO_2 removal, conversion or storage (Mikkelsen et al., 2010). Among them, biological carbon fixation emerged as a very promising technology, thanks to the possibility of working in mild conditions of temperature and pressure and without using chemical substances (Alitalo et al., 2015; Lee et al., 2012).

The combination of CO_2, derived -for instance- from biogas, with H_2 produced from RE sources, and its conversion to CH_4, is, therefore, a valuable solution for both reducing CO_2 emissions and upgrading the biogas to higher CH_4 content. This technology is named ex-situ biogas upgrading and consists in the injection of biogas and H_2 into an anaerobic reactor, where a consortium of mixed hydrogenotrophic culture couples the CO_2 contained in the biogas with H_2, converting it to CH_4, via hydrogenotrophic methanogenesis (Kougias et al., 2016a). Moreover, as explain in section 1.3.2, ex-situ biogas upgrading offers an effective alternative to main limitations related to in-situ biogas upgrading technology (section 2.1), opening to the possibility of biogas upgrading in dedicated reactors.

4.2 Four novel up-flow reactors for efficient ex-situ biogas upgrading

In paper III, H_2 mass transfer rate has been more largely investigated in four up-flow reactors for ex-situ biogas upgrading. Each setup was composed of a thermophilic up-flow reactor having 290 mm height, 35 mm diameter and 850 mL working volume, inoculated with degassed digestate and supplemented with hydrogenotrophic methanogenic enriched inoculum obtained from an upgrading biogas reactor (Bassani et al., 2015). Required nutrient source was daily provided through fresh degassed digestate. The input gas

mixture was composed of CO_2 and H_2 according to the stoichiometry of hydrogenotrophic methanogenesis reaction (1:4, equivalent to 15 and 60% of the total input gas volume), 23% CH_4, in order to simulate typical biogas composition, and 2% extra H_2 as ground gas, which was expected to remain unutilized. This gas mixture was continuously injected either through multiple stainless steel diffusers combined with alumina ceramic sponge or through an alumina ceramic membrane, having different pore sizes, as shown in Figure 5. The effect of the different H_2 distribution devices on the H_2 gas-liquid mass transfer rate was investigated to increase H_2 uptake by methanogens. Moreover, different input gas flow rate (Q_{IN}) and gas recirculation flow rates (Q_{RC}) were tested to enhance the gas transfer to the liquid phase and, therefore, the conversion of CO_2 and H_2 to CH_4 (Table 2). Multiple diffusers were used in order to increase the porosity, i.e. the sparging surface, of the device, allowing the application of Q_{RC} much higher, compared to previous study (chapter 3), and, thus, avoiding excessive pressures. Moreover, based on results reported in chapter 3, ceramic sponge was applied as a means to increase the contact area between gas and liquid phases.

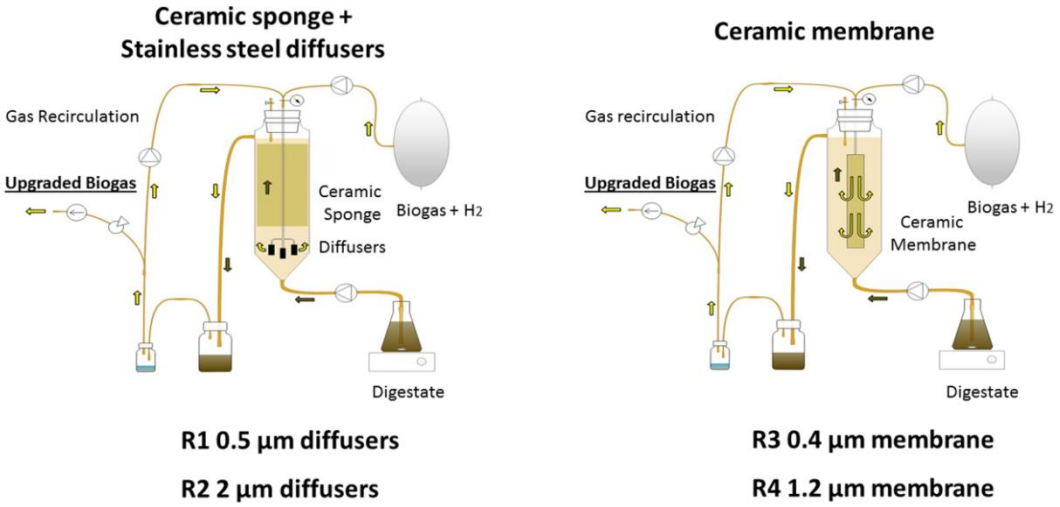


Figure 5: Up-flow reactors configuration (adapted from Paper III).

Table 2: Ex-situ biogas upgrading experimental plan.

Period	GRT (h)	Q_{RC} ($L/L_R.h$)	Q_{IN} ($L/L_R.d$)
I	15	2.88	1.57
II	15	5.75	1.54
III	7	5.75	3.37
IV	7	10.04	3.34
V	4	10.04	5.84
VI	4	20.14	5.84

4.3 Ex-situ biogas upgrading and H_2 mass transfer rate in thermophilic up-flow reactors

Detailed operational data from the reactors are reported in Paper III (Table 2 and Figures 1 and 2). Outcomes of this study stated a rapidly adaptation of the reactors' microbiome to the applied operating conditions and a continued improvement of upgrading performances along all the experimental period. In fact, at the end of the experiment the CH_4 content was improved from 23%, in the input gas, to 96% in the output flow. Moreover, only ~2-4% of the output gas was represented by unutilized H_2. Additionally, CO_2 conversion efficiency (η_{CO_2}) and reactors' CH_4 yield (Y_{CH_4}) reached values higher than 99% and close to the stoichiometric maximum of 0.25 L_{CH4}/L_{H2}, respectively.

Therefore, it can be affirmed that designed reactor configurations and selected operating conditions resulted in efficient biogas upgrading process in terms of CH_4 production, conversion efficiency and output gas quality, generating biogas with 96% CH_4 content, which can be employed as transportation fuel (Deng and Hägg, 2010).

Regarding H_2 to liquid transfer, because of technical issues related to the direct measurement of H_2 in the liquid phase, most of techniques utilize H_2 concentration in the gas phase to estimate the dissolved H_2. Nevertheless, because of the importance of this parameter for the evaluation of H_2 mass transfer, it is essential that the concentration of poorly soluble gases, such as H_2, is directly measured in the liquid phase, rather than being estimated from gas phase (Pauss et al., 1990). In this study, dissolved H_2 has been measured in the liquid phase and H_2 transfer coefficient ($k_L a$) has been calculated, in order to clarify the direct impact of H_2 mass transfer rate on upgrading performances (Paper III (Table 3)). As expected, the concentration of H_2 dissolved (H_{2l}) was markedly lower than its concentration in the gas phase (H_{2gTh}) (i.e. on average 55 times lower). Nevertheless, compared to previous studies (Díaz et al., 2015; Luo et al., 2012; Luo and Angelidaki, 2013b), remarkably higher $k_L a$ values ranging from 105 and 777 h^{-1}were achieved. This is explained by the effectiveness of applied reactor configuration and process conditions to ensure H_2 gas-liquid transfer. The correlation of achieved $k_L a$ values with the other process parameters confirms the importance of H_2 to liquid transfer for its availability for methanogens and thus for ex-situ biogas upgrading process.

4.3.1 Contribution of diffusion device and pore size on upgrading performances

From the analysis of operational parameters, it was shown that the pore size of the diffusion devices influenced the upgrading process, although its contribution was not statistically significant in all the conditions applied. In particular, it was found that larger pore size (R2 and R4) resulted in higher output gas quality, reaching 94.2±1.8% CH_4 content compared to 92.0±2.2% achieved by the smaller pore diameters (R1 and R3). Accordingly, at the end of the experiment, the CH_4 content stabilized to 94 and 96% in R4 and R2, respectively, compared to >92% of R1 and R3. Similarly, despite all configurations performed at very high η_{CO_2} (on average 95%), reactors with larger pore size diffusers showed the best performances (on average 96%). Moreover, H_2 was efficiently utilized during the whole experimental period, with R4 showing the highest H_2 utilization efficiency (η_{H_2}) (>99%). This can be explained by the higher reactor mixing provided by larger pore size devices, compared with smaller ones (Merchuk et al., 1998). In fact, proper mixing speed is known to increase gases $k_L a$ improving gas-liquid contact (Kramer and Bailey, 1991; Luo and Angelidaki, 2012). Consistently, calculation of the $k_L a$ coefficient showed that larger pore size diffusion devices, in particular R2, were able to utilize H_2 providing the highest $k_L a$ (on average 468 h^{-1}). Additionally, R4 presented the best average CH_4 production rate (P_{CH_4}) andY_{CH_4}, while R2 reported the highest values at the end of the experiment (0.82 $L_{CH4}/L_{R.}d$ ($p<0.05$) and 0.23 L_{CH4}/L_{H2}, respectively). Finally, while diffusers' pore size resulted of great importance for upgrading performances, unexpectedly, diffusion device (diffusers and ceramic sponge vs ceramic membrane) did not show significant performance distinctions throughout the experiment.

4.3.2 Contribution of gas recirculation and input gas flow rate on upgrading performances

Notably, in all the reactors increase of gas recirculation rate (Q_{RC}) led to an improvement of upgrading performances, resulting in significant higher CH_4 content and/or P_{CH_4} in several operating condition and reactor configurations. Moreover, as previously reported (Guiot et al., 2011), $k_L a$ increased with higher Q_{RC}; for example, 36% higher $k_L a$ was recorded from period V to period VI. These outcomes state the positive effect of recirculation on gas-

liquid transfer extending the contact time between the two phases and therefore enhancing H_2 availability for microorganisms (Guiot et al., 2011).
Conversely, variations in microbial community living conditions, in this case, excessive Q_{IN} could affect negatively the process resulting in lower performance efficiencies (Kleerebezem and Stams, 2000). In this experiment, a slight decline has been recorded only in Period V, when the highest Q_{IN} was provided. The drop was totally recovered in Period VI, thanks to the application of higher Q_{RC}.

To summarize, the obtained results indicate that larger pore size devices together with a proper gas recirculation flow managed to achieve the most efficient biogas upgrading thanks to the optimal mixing speed and gas retention time. Moreover, the importance of the application in the reactor of a ceramic sponge was confirmed, as it increased gas-liquid contact extending the retention time of gases in the liquid media, therefore increasing H_2 $k_L a$.

5 Bioinformatics approaches for biogas community characterization

5.1 16S rRNA amplicon sequencing (16S AS)

As described in section 1.4, the recent development of NGS technology gave a substantial contribution to the progression of research fields, such as metagenomics. Most of the studies on biogas reactors' microbial community are conducted using 16S AS, where the taxonomic assignment is based on sequence similarity search against reference 16S rRNA sequences deposited in public databases (De Francisci et al., 2015; Luo et al., 2015) (Figure 6). This approach has been applied in Paper III for the analysis of ex-situ biogas upgrading community. From the analysis of 16S AS, 56 operational taxonomic units (OTUs) with relative abundance >0.5% were assigned at different phylogenetic levels and considered for the analysis of the community composition. A description of the microbial population is reported is section 6.4.

5.2 Assembled Full-Length 16S rRNA gene sequencing (16S AFL)

Despite the extensive and established use of 16S AS, analysis limited to a restricted portion of gene can produce ambiguous assignments, due to the high conservation of sequences (Miller et al., 2011). Therefore, in Paper I, full-length 16S rRNA gene has been reconstructed providing more complete taxonomic information. Within this study, rRNA-like sequences were extracted from Total Random Sequences (TRS) and 16S rRNA Shotgun Reads (16S SR) were utilized to calculate alpha diversity indexes and to draw rarefaction curves, through MG-RAST toolkit (Paper I; Meyer et al., 2008). Extracted 16S SR were then aligned to a large 16S rRNA database to reconstruct the most probable full 16S rRNA gene, using EMIRGE (Miller et al., 2011), and taxonomically assigned as described in Paper I and shown in Figure 6. In the present study, this recently developed method was, for the first time, applied to the study of biogas reactors' community and was named 16S AFL. The reconstruction of 16S SR to 16S AFL is independent from reference database completeness and can be performed from unknown reference sequences, calculating the probabilities of errors when mapping the reads against the reference. At each interaction between reads and reference, reference sequence is corrected according to the probability achieved. Therefore, correct reference sequences and reads abundance progressively adjust and finally

stabilize. Moreover, when reference sequences become similar they are merged together, while, if different reads are mapped onto the same reference, the reference is split into two different sequences. In this way, the reference database works as potential initial sequence and the probability that each base is correct changes at each iteration and the base with the highest probability is chosen at each position (Miller et al., 2011).
In paper I, from the reconstruction of 16S rRNA gene into 16S AFL, on average 70 sequences per sample were obtained. Because only the sequences of most abundant microorganisms are expected to be assembled, achieved 16S AFL are attributed to the most relevant members of the community. A description of the microbial population, obtained with this strategy, is provided in section 6.2.

5.3 Total Random Sequencing (TRS)

Due to the complexity of the AD community and being the most of biogas reactor's population uncharacterized (Albertsen et al., 2013), single gene sequencing analysis applied in Paper I reached, in some cases, a low phylogenetic resolution. Therefore, the composition of the microbial community was further determined from TRS, using metagenomic phylogenetic analysis (MetaPhlAn) tool, as described in Paper I and shown in Figure 6. This tool estimates the relative abundance of microorganisms by mapping TRS against unique clade-specific marker genes that can unambiguously identify specific microbial clades until species level (Segata et al., 2012). Precisely, MetaPhlAn compares each TRS with a marker genes' database selecting high-confidence matches, thanks to the uniqueness of available markers. Moreover, this tool normalizes the number of TRS matching with each clade by the markers' nucleotide length providing the relative abundance of each taxonomic unit (Segata et al., 2012). From the application of this strategy an exceptionally rich profile of microbial community was depicted revealing, for instance, numerous phyla not detected by 16S AFL analysis. A description of the microbial community according to TRS analysis is reported in section 6.2.

5.4 *De novo* assembly and binning strategy

Even in TRS analysis, most of reference genomes used to phylogenetically classify the microbial communities are isolated from diverse environments, where only a small fraction of microorganisms have been cultivated (Albertsen et al., 2013). Therefore, the taxonomic assignment performed on TRS might remain, for some extent, uncertain. Previous works demonstrated

that by assembling TRS and following a binning strategy, it was possible to acquire a clearer insight into the biogas microbial community (Campanaro et al., 2016).

In Paper IV and V, *de novo* assembly of TRS generated in Paper I has been applied creating a large set of scaffolds, which have been successively clustered in individual genomes with a procedure called binning (Figure 6). Binning can be performed with different criteria, but in these studies the classification was based on the rationale that scaffolds belonging to the same genome are found with the same relative abundance and are attributable to the same microorganism. The phylogenetic assignment of each biological entity, defined genome bin (GB), was performed with two independent software: Phylophlan and Phylopythia. The first approach identifies hundreds of conserved proteins from the current catalogue of microbial genomes and uses, as input, all protein sequences belonging to each GB to provide a complete high resolution phylogeny (Segata et al., 2013). Conversely, the second method uses the sequence composition of all the scaffolds assigned to each GB to phylogenetically characterize the input sequences (McHardy et al., 2007).

Besides the determination of biogas upgrading microbiome and the effect of H_2 on its composition, this research aimed to define the core microbial consortium of biogas reactors independently from the operating conditions. This was possible by comparing GBs generated from this study with the ones generated from a previous study on biogas reactors' communities (Campanaro et al., 2016; Kougias et al., 2016b). Identification of the common GBs was performed determining the Average Nucleotide Identity (ANI) of the protein-encoding nucleotide sequences. Two GBs were attributed to the same species if at least 50% of the genes was matching and if the average nucleotide-level similarity was >95% (Paper IV). This study led to the most accurate insight into biogas microbial community generating 236 GBs, of which 157 were newly identified. Moreover, from the comparative study conducted on previously identified GBs, it was possible to recognize a subgroup of common microorganisms that could represent the essential core of biogas community. A detailed description of the microbial community is reported in sections 6.2 and 6.3. Interestingly, most of the GBs identified were characterized at low taxonomic level confirming that biogas microbial consortium is still mainly composed of unknown species.

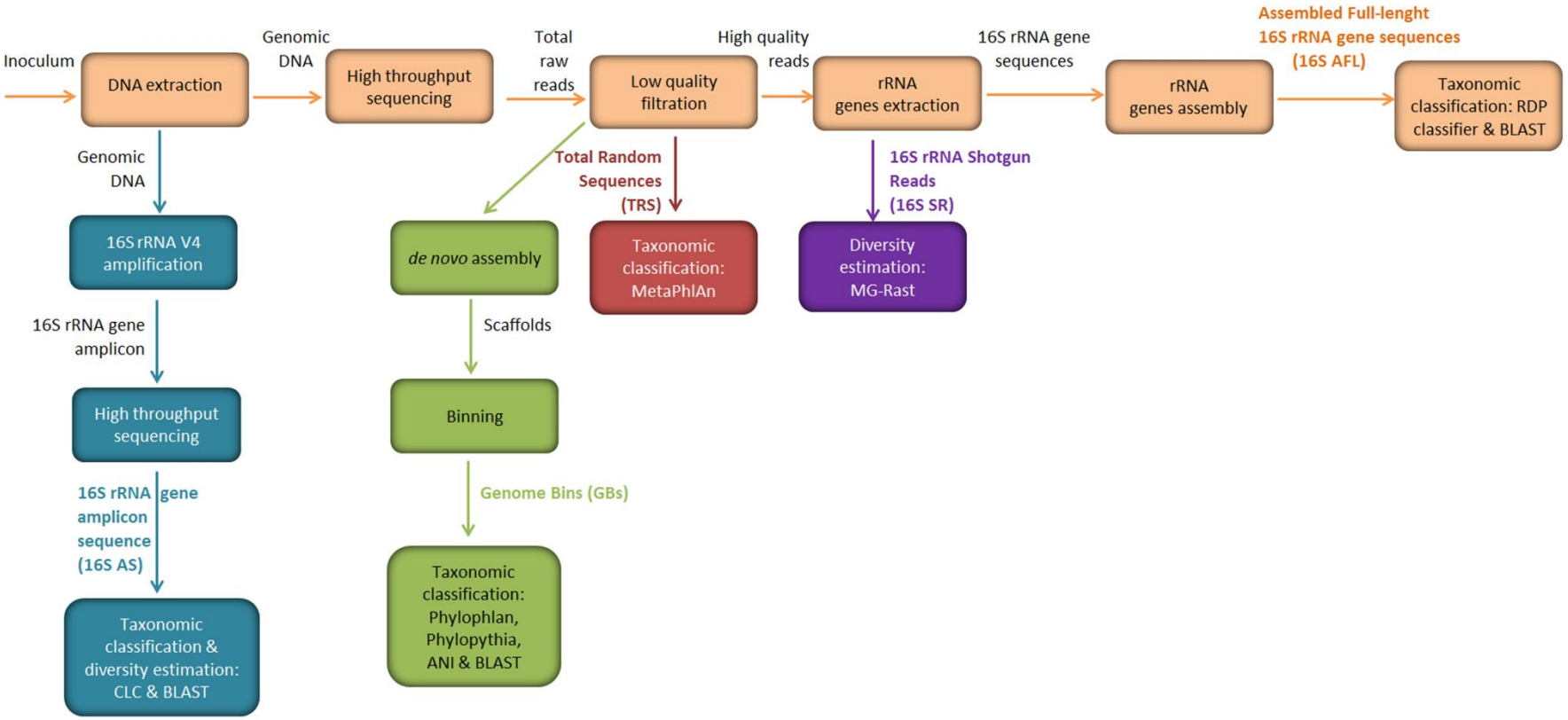


Figure 6: Flowchart representing the main steps of bioinformatics analysis for biogas microbial community characterization.

6 Microbial community characterization and effect of hydrogen on community composition

6.1 Importance of community characterization for efficient biogas production and upgrading

As described in section 1.2, biomethanation occurs through a series of reaction attributed to a complex and mainly unidentified microbial consortium which activity and interaction are crucial for efficient process operation.

Previous studies demonstrated that in anaerobic reactors there is a fraction of microorganisms always present and constituting the core of the community and another fraction of microorganisms that depend on operating conditions and sludge source (Rivière et al., 2009). Nevertheless, such a core community for biogas reactors in not established, yet. Therefore, a deeper insight into biogas community composition is fundamental to better understand and optimize AD process, but also to define the impact of different operating condition on the community. For this aim, in Paper IV, the core microbiome of biogas reactors has been elucidated through a comparative study between the newly described community and previously investigated biogas consortia (Campanaro et al., 2016). Furthermore, in Papers I, III, IV and V, a particular focus was given to the study of the unexplored biogas upgrading microbial community. The interest is motivated by the hypothesis that H_2 addition changes the community composition promoting the hydrogenotrophic methanogenic pathway and, thus, the CO_2 consumption, enhancing biogas upgrading.

6.2 Microbial community shift upon H_2 addition in biogas upgrading reactors

Microbial community populating the secondary stage of mesophilic and thermophilic reactors, before and after the H_2 addition, described in Paper I, has been analyzed with different bioinformatics approaches (chapter 5) and a detailed picture of the microbial community is reported in Figures 7 and 8 and Papers I (Figures 2 and 3), IV (Figures 1 and S1) and V (Figure 1). Moreover, the impact of H_2 addition on the community composition has been investigated and is shown in Figure 8 and Papers I (Figure 2) and V (Figure 1).

Interestingly, mesophilic reactor was characterized by higher diversity, compared to the thermophilic. Nevertheless, upon H_2 addition, the diversity decreased at both temperature conditions resulting into a more specialized community. In accordance with previous studies, *Firmicutes, Bacteroidetes* and *Proteobacteria* were the most abundant phyla. This can be explained by their role in the hydrolysis of polysaccharides and proteins contained in cattle manure (De Francisci et al., 2015; Krakat et al., 2011). Additionally, members of *Firmicutes* and *Proteobacteria* are also involved in acetogenesis and syntrophic VFA degradation (Krakat et al., 2011).

Phylogenetic assignment performed with different approaches showed the occurrence of diverse microbial groups. Specifically, among *Firmicutes*, *Clostridiales* was the most prevalent phylogenetic order. Taxonomic classification of reconstructed GBs assigned to this order 126 of the 141 GBs attributed to phylum *Firmicutes.* Among them, the majority of GBs identified at family level was assigned to family *Syntrophomonadaceae* (46 GBs), populating mainly the thermophilic reactor. In general, H_2 addition did not evidently impact on the growth of these microorganisms, displaying only few GBs markedly changing in abundance. However, the behavior of most interesting microrganisms will be further discussed in this section. In general, *Clostridiales* are known to play an important role for cellulosome-mediated cellulose hydrolysis; however they do not present the β-sugar consumption pathway. Therefore, this reaction is carried out by *Thermotogales* and *Sphingobacteriales* in syntrophic relation with *Clostridiales* (Jiménez et al., 2014; Xia et al., 2014). This can explain the co-occurrence and dynamicity of these microorganisms with some members of *Clostridiales* observed in this study. Among less represented families, interestingly, a GB assigned to *Pelotomaculum* was found at thermophilic conditions and decreasing >8-folds upon H_2 addition. Syntrophic relationships of *Syntrophomonas* and *Pelotomaculum* with identified methanogens are acknowledged and could explain the presence of these microorganisms mostly in thermophilic samples and the parallel behavior of some of them upon the H_2 addition (Tatara et al., 2008; Tischer et al., 2013; Wirth et al., 2012). Moreover, *Halanaerobiaceae*, were found to be thermophilic and represented by 2 GBs. In particular a *Halothermothrix* was positively correlated to H_2 addition (2-folds). Within *Erysipelotrichaceae*, 6 of the 8 GBs identified were assigned at species level to *Erysipelothrix rhusiopathiae*. In general, they were low abundant and did not show appreciable distinction between the two temperature conditions applied. Moreover, their occurrence was found to decrease upon H_2 addition (on average 3-folds). Finally, 16S AFL analysis identified microorganisms simi-

lar to genera *Halocella* and *Sedimentibacter* and species and *Tissierella creatinini* (95% identity).

The most of *Bacteroidetes* were assigned to order *Bacteroidales*, while only a minor fraction was assigned to *Flavobacteriales*. In general, they mainly populated the mesophilic community and either increased or decreased upon H_2 addition. The occurrence of this phylum mainly at mesophilic condition can be explained by the presence in their genomes of a low number of genes for stress resistance. The most representative members of this phylum were 2 GBs assigned to *Rikenellaceae* and *Alistipes* (157 coverage (cov)) and an unclassified *Sphingobacteriaceae* accounting for 15% of the mesophilic community, before the H_2 addition, and decreasing after H_2 addition. The decrease of *Rikenellaceae* and *Porphyromonadaceae* in concomitance with *Erysipelotrichaceae* can be explained by the role of the formers in acetic and propionic acid production and the consequent utilization of these compounds by the latter (Hahnke et al., 2015; Stolze et al., 2015; Su et al., 2013). Moreover, at thermophilic conditions, *Cytophagales* was found with >18% relative abundance. The high occurrence of these microorganisms, before the H_2 addition can be due to their function in synergistic cellulose degradation (Hung et al., 2011). Genera such as *Bacteroides, Cellulophaga,* and *Flavobacterium* were also detected. Their function is mainly related to macromolecules fermentation and consequent acids, CO_2 and H_2 production, thanks to the presence of enzymes for polysaccharides' and proteoglycans' cleavage (Hanreich et al., 2013; Traversi et al., 2012; Wang et al., 2014).

As a general trend, *Proteobacteria* abundance decreased on average 3-folds. Among them, 2 GBs assigned to *Alcaligenaceae* and *Moraxellaceae* decreased up to 34-folds. Conversely, 3 GBs, such as a member of *Xanthomonadaceae*, showed the opposite behavior, increasing up to 95-folds. Interestingly, family *Pseudomonadaceae*, genus *Advenella* and less abundant phyla *Actinobacteria* and *Tenericutes* showed a concordant decrease upon H_2 addition (4-folds) and are known to be involved in recalcitrant compounds decomposition producing enzymes for lignocellulose degradation (Boucias et al., 2013; Ntougias et al., 2013). Among them, notably, one *Actinobacteria* was assigned to *Corynebacterium humireducens* str. DSM 45392 (Average Nucleotide Identity (ANI) >97%). According to TRS, relevant species such as *Desulfobulbus propionicus* and *Desulfurivibrio alkaliphilus* were also detected. The former decreased at both temperature conditions, the latter, coherently with the higher pH observed, increased at thermophilic conditions. The decrease of *D. propionicus* can be related to its ability to use H_2, in absence of sulfate, to convert acetate and CO_2 to propionate (Laanbroek et al.,

1982). Therefore, the stimulation of hydrogenotrophic methanogens could have caused a competition for the H_2. Moreover, several species of *Desulfovibrio* were identified, with *D. desulfuricans* as most abundant microorganism. Under sulfate limited conditions, these species can produce acetate, H_2 and CO_2 in co-occurrence with hydrogenotrophic methanogens (Bryant, 1979; Muyzer and Stams, 2008). Therefore, in the thermophilic reactor, the concomitant higher abundance of *Desulfovibrio* spp. (>1-fold) and hydrogenotrophic methanogens are indicative of a potential syntrophic association between these species that could be of great relevance for biogas production and upgrading. Finally, a minor fraction of *Proteobacteria* was represented by species *Arcobacter butzleri* and genus *Campylobacter*.

Less abundant phyla were also characterized. Among them, *Lentisphaerae* and *Synergistetes* were present at both temperature conditions. Notably, 2 members of *Synergistetes* were assigned to *Anaerobaculum* and *Aminobacterium colombiense* str. DSM 12261(ANI 99.5%). Interestingly, these two phyla showed an opposite behavior upon H_2 addition; while *Lentisphaerae* increased up to 5-folds, *Synergistetes* decreased up to 3-folds. Functional annotation of *Lentisphaerae* GB indicates its capability for monosaccharides and polysaccharides degradation. Nevertheless, no pathways for the interspecies H_2 transfer were found. Besides, the low abundance of *Synergistetes* and its general decrease after H_2 addition can be explained by *A. colombiense* ability in H_2 production. In fact, the higher P_{H2} might have played a suppressive effect on these microorganisms (Chertkov et al., 2010). Moreover, consistently with *Fibrobacter succinogenes* ability to use H_2 as electron donor in fumarate reduction (Suen et al., 2011), 1 GBs assigned to this species increased up to 30-folds upon H_2 addition. Similarly, 16S AFL analysis revealed the presence of a microorganism assigned to *Candidatus* Cloacamonas acidaminovorans (92% identity) accounting for 1.7% of the mesophilic community, upon H_2 addition. This newly discovered bacterium ferments sugars and amino acids and oxidize propionate to H_2 and CO_2 indicating this species as a potential syntrophic bacterium (Pelletier et al., 2008; Stolze et al., 2015). Finally, the low occurrence of *Chloroflexi* and *Actinobacteria* indicates that the structure of the microbial community populating biogas reactors treating agricultural and industrial residues is highly different compared to AD systems processing sludge and wastewater. In fact, it is known that members of these phyla dominate the community in activated sludge systems (Albertsen et al., 2015; Wang et al., 2014), as they are aerobic or facultative anaerobic microorganisms growing in the influent feed. There-

fore, in strictly anaerobic environments, such as the biogas reactors, the proliferation of these phyla is not favored.

According to unassembled TRS analysis, phylum *Euryarchaeota* represent one of the dominant phyla, which relative abundance increased upon the H_2 addition from 17 to 45% (~3-folds) and from 27 to 36%, in mesophilic and thermophilic reactor, respectively. The different bioinformatics approaches applied allowed the identification of various archaeal genera and species. Among them, interestingly, relative abundance of a species similar to *Methanoculleus marisnigri* (>97% identity) increased from 8 to 36% (4.5-folds) in the mesophilic reactor and from 17 to 24% (1.5-folds) in the thermophilic. A recent metagenomic study focussing on this methanogen confirmed that this is a hydrogenotrophic species and it was provisionally named as *Candidatus* Methanoculleus thermohydrogenotrophicum (Kougias et al., 2017). Moreover, other 2 archaeal GBs were reconstructed and assigned to *Methanoculleus thermophilus* and *Methanothermobacter*; notably, they were found mainly at thermophilic conditions. This behavior is expected because both GBs are taxonomically related to species well adapted to high temperature (Narihiro et al., 2016; Liu and Whitman, 2008; Wasserfallen et al., 2000). Moreover, *Methanoculleus* and *Methanothermobacter* are known to be dominant in AD communities populating reactors processing manure substrates (Campanaro et al., 2016; Kröber et al., 2009; Luo and Angelidaki, 2013b). Interestingly, the occurrence of these GBs in response to temperature and H_2 was heterogeneous and characteristic for each one. In fact, at mesophilic conditions, they were respectively increasing and decreasing (3-folds). On contrary, at thermophilic conditions, they were respectively decreasing (92-folds) and increasing (4-folds). This can be expected, because methanogens are known to have different affinities for the H_2, resulting in competition for the substrate (Tang et al., 2011). Moreover, this heterogeneity is important to maintain a homeostatic CH_4 production among different conditions. In fact, the activity of a specific methanogen can be replaced by another one better adapted to the new incoming condition. Additionally, a remarkable finding was the assignment of a GB to a newly discovered member of *Methanomassiliicoccales* (Campanaro et al., 2016). Finally, TRS showed the occurrence of the known hydrogenotrophic methanogens (Garcia et al., 2000; Oren, 2006) *Methanocorpusculum labreanum*, *Methanogenium* sp., (1−3%) and an unknown genus of *Methanoregulaceae* (6−7%).

Regarding aceticlastic population, *M. mazei* was found with >1% relative abundance while *M. barkeri* and *M. acetivorans* were less represented. In general, aceticlastic methanogens had similar abundance at both temperature

conditions, showing a high ability in adaptation to temperature fluctuations. The low abundance of genus *Methanosarcina* can be explained by the low VFA and acetate concentration in the reactors. In fact, high acetate levels are known to selectively stimulate the proliferation of aceticlastic methanogens (Wirth et al., 2012). Moreover, aceticlastic methanogens are more sensitive to pH and ammonia levels and high P_{H2} than hydrogenotrophic (Ahring et al., 1991; Oren, 2006). The decrease of *M. mazei* (8 and 1.5-folds at mesophilic and thermophilic conditions, respectively) was likely compensated by the increased abundance of mentioned hydrogenotrophic methanogens. Additionally, in absence of aceticlastic methanogens, acetate consumption and CH_4 formation can be carried out through SAO and reduction of CO_2 and H_2 to CH_4 by hydrogenotrophic methanogens (Schnürer et al., 1999).

Considering genes involved in the WLP, 14 GBs presented the whole or nearly the whole pattern of genes (≥8) involved in this pathway. Nevertheless, their presence did not necessary demonstrate the actual activity of this pathway in the direction of acetate oxidation (Cord-Ruwisch et al., 1988; Winter and Wolfe, 1980; Zinder and Koch, 1984). 6 of these GBs also had a high number of genes (≥20) for butyrate pathway and one had >20 genes for propionate pathway (Table 3 in Paper V). Interestingly, comparison of mentioned GBs with the sequenced genomes of the NCBI database revealed that most of them were not previously known at species level. This finding is of particular interest because the discovery of potentially uncharacterized SAO bacteria represents the starting point to unveil their unknown functions. Remarkably, 12 of these 14 GBs belonged to the *Synthrophomonadaceae* family, while one was assigned to *Tepidanaerobacter*, possibly related to *Tepidanaerobacter acetatoxydans*, a well-known SAO bacterium (Westerholm et al., 2011). This GB was found at thermophilic conditions and decreasing 5-folds upon H_2 addition. In general, within the analyzed communities, 2 other GBs were assigned to *Thermoanaerobacteraceae* family. Interestingly, each of them was occurring only at one of the two temperature conditions applied, and they were both increasing upon H_2 addition (up to 3-folds) reaching a coverage value of 98. Consistently, according to 16S AFL, a very abundant 16S rRNA gene sequence, increasing to >20% and >7% at mesophilic and thermophilic condition, respectively, was assigned to this family.

Notably, 11 of the 14 GBs mentioned were mainly thermophilic, 2 were mesophilic and 1 was equally present at both temperature conditions. This is expected because, at higher temperatures, SAO pathway is less energetically unsustainable and, thus, SAO bacteria may compete with aceticlastic meth-

anogens for the substrate (Schink, 1997). Addition of H_2 did not strongly impacted on their abundance, as only the 2 mesophilic GBs (*Syntrophomonadaceae* sp. DTU232 and *Clostridia* sp. DTU204) and the thermophilic *Tepidanaerobacter* DTU063 markedly decreased (up to 25-folds) and 2 other GBs slightly increased. This behavior is unexpected and of particular interest, because the increase of P_{H2}, consequent to the introduction of external H_2, should make acetate degradation less sustainable because SAO activity can be energetically feasible only if the methanogenic partners keep P_{H2} low. A possible explanation is that, when P_{H2} is not low enough to make SAO reaction sufficiently exergonic, these microorganisms might utilize the WLP to form acetate (homoacetogenesis) rather than consume it (SAO).

Another pathway utilized by bacteria for syntrophic H_2 transfer to methanogens is the β-oxidation of butyrate. During this reaction, microorganisms such as *Synthrophomonadaceae* species (e.g. *Syntrophomonas wolfei*) produce H_2 from the reoxidation of reducing equivalents (NADH and reduced electron transfer flavoprotein) (McInerney and Bryant, 1981; Wallrabenstein and Schink, 1994). 12 GBs presented ≥20 genes (p value < 0.05) for both propionate and butyrate degradation. Nevertheless, they were phylogenetically different from SAO bacteria previously described and were assigned to family *Alcaligenaceae* and other members of *Proteobacteria*. Moreover, other 6 and 9 GBs showed a high number of genes (≥20) exclusively for propionate and butyrate, respectively. GBs having genes only for propionate degradation were more phylogenetically heterogeneous; conversely, 11 out of the 21 GBs presenting a high number of butyrate degrading genes belonged to *Syntrophomonadaceae* family. As expected, 16 out of 21 of the butyrate-degrading species showed also numerous genes for fatty acid degradation (≥10), indicating that generation of reducing equivalents occurred through β-oxidation. Moreover, 11 of the 12 GBs having an enriched number of genes for both propionate and butyrate degradation presented genes for menaquinone biosynthesis or binding (Table 3 in Paper V). This finding is of particular interest because, in obligate syntrophic species, propionate and butyrate metabolism is coupled with menaquinone reduction, resulting in protons movements and consequent H_2 transfer to the syntrophic archaea (Stams and Plugge, 2009). Conversely, most of the GBs presenting genes for the WLP did not show genes for menaquinone synthesis, indicating that these microbes use a different process to transfer H_2 to methanogenic archaea. Interestingly, H_2 addition impacted more negatively on butyrate degraders than on GBs with WLP, as 8 out of 12 decreased in abundance upon

H_2 addition. This could be explained by the negative effect of the higher P_{H2} on these syntrophic bacteria or by the decreased level of butyrate observed in the reactors after H_2 addition.

In conclusion, at both temperature conditions aceticlastic methanogens decreased, although more markedly at mesophilic conditions. The concomitant increase of *Methanoculleus*, *Methanothermobacter* and *Mehanoregulaceae* confirms the role of the H_2 in the selective stimulation of the hydrogenotrophic pathway and the consequent suppression of the aceticlastic. Consistently, in general, bacteria involved in the first steps of the AD process decreased in abundance, while syntrophic bacteria such as *Desulfovibrio*, *C. acidaminovorans*, and some members of *Thermoanaerobacteraceae* and *Syntrophomonadaceae*, increased. These findings, together with the decreased microbial diversity, upon H_2 addition, state the role of the H_2 moving the AD process toward the final steps stimulating CO_2 consumption and therefore biogas upgrading.

6.3 Toward the definition of a core community for biogas production: a comparative study

In paper IV, GBs extracted from biogas production and upgrading communities at mesophilic and thermophilic conditions and from a previously investigated community (biogas production at thermophilic conditions) were compared, to determine similarities and differences in GBs phylogenetic distribution. A detailed description of the two communities is reported in Figures 7 and 8 and Paper IV (Table 2 and Figures 1, S1 and S2). Based on the hypothesis that H_2 addition would promote specific microbial groups (enhancing, for instance, the hydrogenotrophic methanogenic pathway), the specific composition of the microbial communities is attributable to environmental and operating conditions. Conversely, a potential core community, required for biogas production, could be identified in the common microbial groups, found in all the studied conditions.

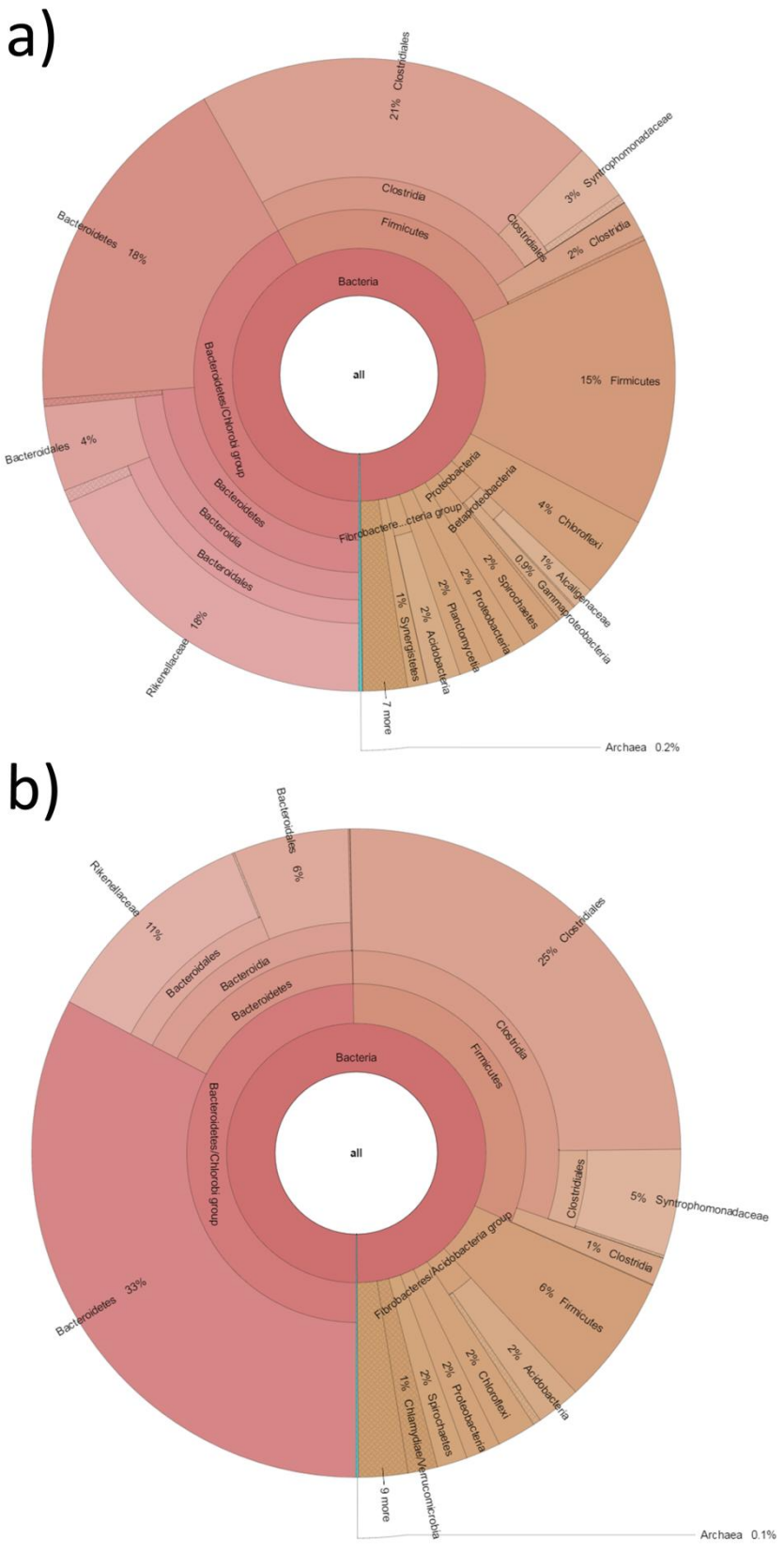
From the previously analyzed community, 106 GBs were extracted. The currently analyzed community included 71% of previously identified GBs (Campanaro et al., 2016). Moreover, 159 new GBs were identified. From the analysis of all 265 unique GBs, the common GBs represented the 29% of the total, while 60% were specific of the current community. Notably, the 67% of archaeal population was common to the two communities. Specifically, 1 GB assigned to *Methanothermobacter* and 2 GBs identified as *Methanoculleus* were present in both assemblies, while 1 GB assigned to *Methanosarcina* was

newly identified. The resilience of the archaeal community can be explained by their crucial role in methanogenesis. Moreover, their independence from the influent substrate can be explained by the fact that they utilize, as substrates, products derived from last steps of the process, which are a limited set of molecules independent from the initial substrate. Moreover, resilience of syntrophic bacteria such as *Syntrophobacterales* and *Synergistia*, can be motivated by their relation with methanogens and their specialised function in AD process (Werner et al., 2011).

Conversely, as observed in the previous assembly (Campanaro et al., 2016), fermentative bacteria belonging to class *Clostridia* and phylum *Bacteroidetes* (section 1.2) resulted more abundant and more diverse. This can be explained by their role in initial methanogenesis steps, which require the concomitance of multiple microbial groups to ensure efficient substrate degradation (Campanaro et al., 2016). Additionally, these microorganisms resulted remarkably dependent on operating conditions. For instance, the different temperatures applied in the current community analysis deeply influence fermentative bacteria composition. In fact, only 17% of *Bacteroidetes* and 14% of *Proteobacteria* were in common with the previously analyzed thermophilic community. Due to the heterogeneity of phylum *Firmicutes*, which include both fermentative and syntrophic bacteria, the 50% of the members was common to the two communities. However, by excluding *Syntrophomonadaceae* family, known for its resilient syntrophic activity with methanogens (section 1.2), the percentage of common GBs decrease to 36%.

Regarding the impact of operating conditions on community structure, mesophilic conditions and H_2 addition are considered crucial parameters determining the most of dissimilarities enlightened by the current analysis, compared to the previous one. Specifically, mesophilic conditions enhanced the proliferation of specific phyla only slightly detected at higher temperatures: *Acidobacteria*, *Chloroflexi*, *Fibrobacteres* and *Planctomycetes*. Most of these microorganisms are known to be involved in the conversion of organic matter, aromatic compound and acetate fermentation (Krakat et al., 2011). Moreover, *Planctomycetes* are known for their ability to oxidize ammonium and for their extremely slow growth rate (Krakat et al., 2011), therefore the higher HRT typical of mesophilic reactor operation could have favored their proliferation. Interestingly and in accordance with previous studies, most of *Bacteroidales* were found in higher abundance in mesophilic samples (Li et al., 2014a). The presence of microorganisms detected mainly at mesophilic conditions is supported by earlier analysis on 16S SR extracted from the same community showing higher diversity in mesophilic samples. Moreover, H_2 addition was

the main responsible for the proliferation of *Syntrophomonadaceae* family, due to their co-occurrence with hydrogenotrophic methanogenic archaea.
To summarize, despite the differences observed between the two assemblies, biogas production core community can be considered as composed of several recurrent microbial groups, including resilient methanogenic archaea such as *Methanoculleus* and *Methanotermobacter* and bacteria belonging to phylum *Proteobacteria* and genus *Synthrophomonas*.


a)
21% Clostridiales
Clostridia
Firmicutes
3% Syntrophomonadaceae
Clostridiales
2% Clostridia
Bacteroidetes 18%
Bacteria
all
15% Firmicutes
Bacteroidetes/Chlorobi group
Bacteroidales 4%
Bacteroidetes
Bacteroidia
Bacteroidales
16%
Rikenellaceae
Proteobacteria
Betaproteobacteria
Fibrobactere...cteria group
4% Chloroflexi
1% Alcaligenaceae
0.9% Gammaproteobacteria
2% Spirochaetes
2% Proteobacteria
2% Planctomycetia
2% Acidobacteria
1% Synergistetes
7 more
Archaea 0.2%
b)
Bacteroidales 6%
Rikenellaceae 11%
Bacteroidales
Bacteroidia
Bacteroidetes
25% Clostridiales
Clostridia
Firmicutes
Bacteria
all
Bacteroidetes/Chlorobi group
Clostridiales
5% Syntrophomonadaceae
1% Clostridia
Fibrobacteres/Acidobacteria group
6% Firmicutes
Bacteroidetes 33%
2% Acidobacteria
2% Chloroflexi
2% Proteobacteria
2% Spirochaetes
1% Chlamydiae/Verrucomicrobia
9 more
Archaea 0.1%

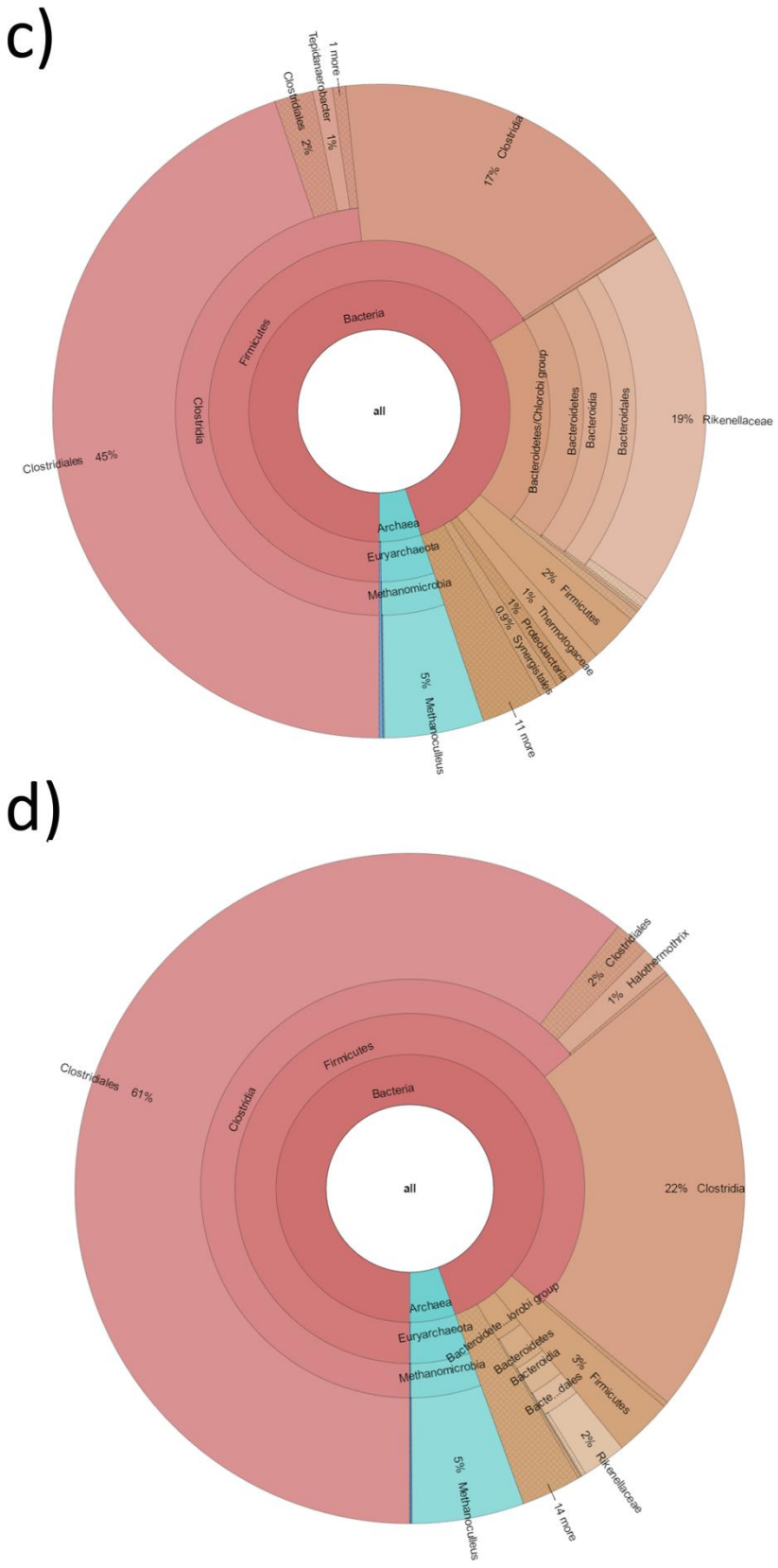


Figure 7: Graphical representation of the microbial community depicted by *de novo* assembly and binning strategy, at the different conditions applied: a) mesophilic pre

H_2, b) mesophilic post H_2, c) thermophilic pre H_2 and d) thermophilic post H_2. The percentages refer to the number of GBs assigned to each taxonomic group.

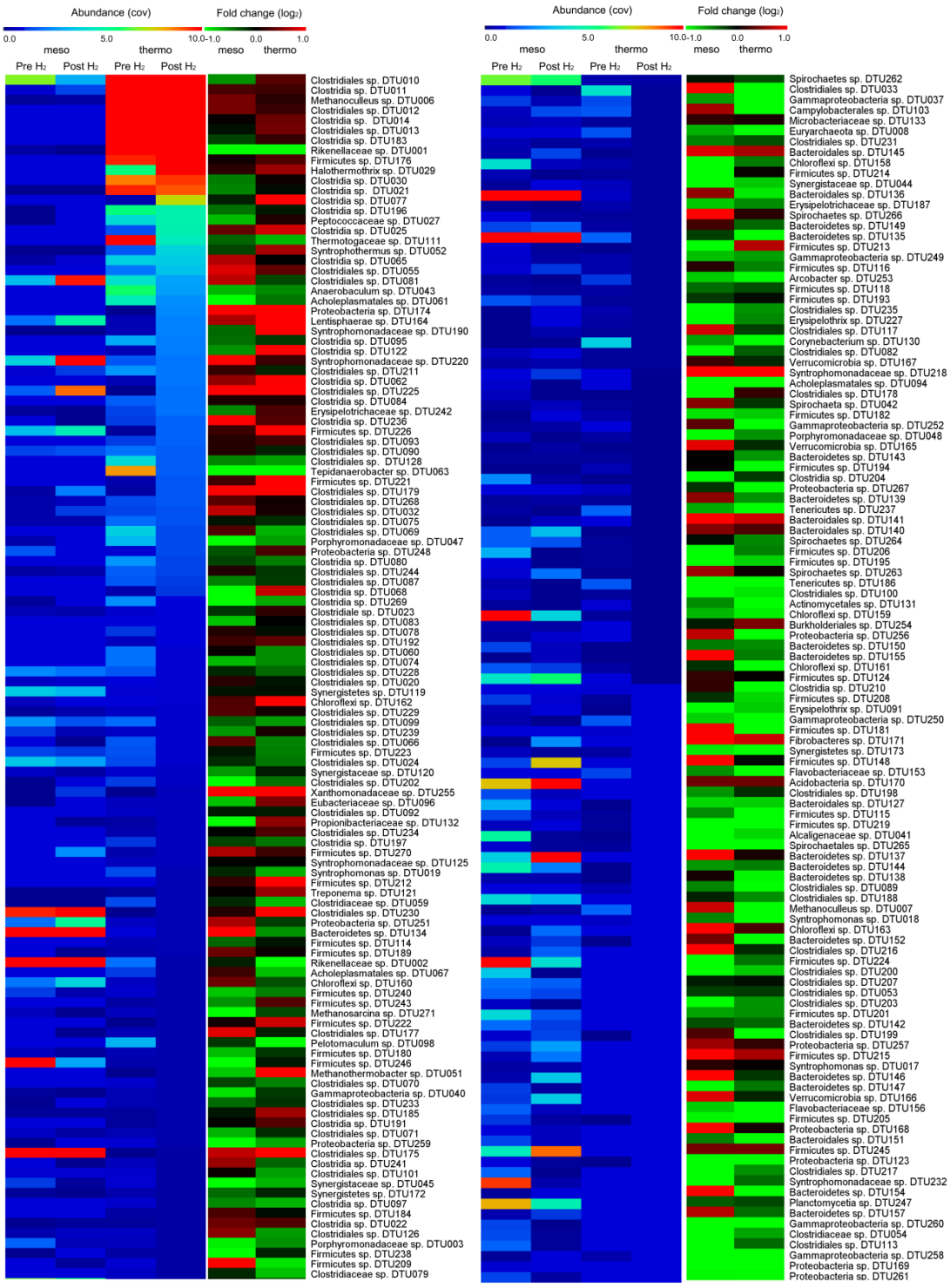


Figure 8: Heat maps of abundance (cov; left part of each panel) and folds change (log_2; right part of each panel) of the GBs reconstructed from mesophilic and thermophilic communities at steady state before and after the H_2 addition. Correspondence between colors and abundance or folds change is reported in the scale at the top of each panel. Folds change is represented in red and green for increased and decreased microorganisms, respectively.

6.4 Ex-situ biogas upgrading microbial community composition

Analysis of 16S rRNA gene sequencing was carried out on thermophilic reactors' communities performing ex-situ biogas upgrading, as described in Chapter 4, and the outcome of the analysis is shown in Paper III (Figure 3). The results showed that, in accordance with previous studies, *Firmicutes* represented the most abundant phylum accounting for the 32% of the microbial community (Campanaro et al., 2016). Interestingly, *Synergistetes*, with *Anaerobaculum mobile* as most abundant OTU (15% of the community), was the second most abundant phylum, which relative abundance doubled to >26% at the end of the experiment. Other 2 OTUs assigned to the same phylum were found to increase up to 40-folds. Occurrence of *Synergistetes* has been previously observed in reactors used for biogas upgrading (sections 6.2 and 6.3) and is consistent with their role and resilience in AD process (Campanaro et al., 2016; Werner et al., 2011). Moreover, analysis of KEGG and COG functional categories showed that *Anaerobaculum* is involved in amino acids and carbohydrates metabolism and the presence of genes involved in WLP and its concomitant increase with *Methanothermobacter thermautotrophicus* indicates a possible role of *A. mobile* in SAO activity with this methanogen.

In accordance with a recent study on ex-situ biogas upgrading, the second most abundant OTU, accounting for ~15% of the community, was assigned to the newly discovered order MBA08 (class *Clostridia*). This is indicative of a remarkable resilience of this microorganism to the operating conditions suggesting its possible involvement in syntrophic relations with hydrogenotrophic methanogens (Kougias et al., 2016a). Moreover, BLASTn search against NCBI database revealed a similarity to the newly identified *Hydrogenispora ethanolica* (90% identity). Nevertheless, being the sequence identity score lower than the threshold for genera classification, the taxonomy of this OTU remains uncertain indicating this microorganism as a new species.

Proteobacteria represented the third most abundant phylum increasing from 0.1 to 11% of the community (131-folds; $p<0.05$). In particular, OTUs were assigned to genera *Campylobacter* (>5%), *Pseudomonas* and *Arcobacter* and species *Advenella faeciporci* and *Dechloromonas agitata*. These genera have been previously found in biogas producing and biogas upgrading reactors (sections 6.2 and 6.3) and, according to COG and KEGG analysis, they are involved in polymers and monomers metabolism. On contrary, relative abundance of phylum *Bacteroidetes* dropped by 3-folds (from 21 to 7%) probably

due to the effect of the H_2 on the community composition, not promoting fermentative bacteria.

Similarly to results described in section 6.2, 10 OTUs assigned to members of *Thermoanaerobacterales*, mostly accounting for >1% of the community and increasing at the end of the experiment, were found. Two of them were identified as genus *Thermacetogenium* and species *Tepidanaerobacter syntrophicus*. As shown by COG and KEGG analysis, genes involved in WLP suggest the role of these microorganisms in SAO pathway (Treu et al., 2016a; Westerholm et al., 2011). Additionally, the significant increase of OTUs assigned to *Syntrophomonadaceae* (~30 folds; $p<0.05$) can be related to their syntrophic relation with hydrogenotrophic methanogens (Treu et al., 2016a and b).

Conversely, members of *Clostridiales* were found either to increase or to decrease at the end of the experiment. More specifically, genus *Clostridium* and species *Lutispora thermophila* increased (<46-folds; $p<0.05$), while *Caldicoprobacter* and *Tepidimicrobium xylanilyticum* decreased (on average 8-folds; $p<0.05$). These microorganisms have been simultaneously found at thermophilic conditions and to degrade proteins for VFA production (Lee et al., 2016; Li et al., 2014b; Tang et al., 2011). Moreover, as shown by predictive COG and KEGG analysis, *Clostridium* and *Caldicoprobacter* are mainly involved in carbohydrates and sugars metabolism and *Clostridium* can also perform SAO activity showing genes for WLP.

Regarding *Archaea* domain, phylum *Euryarchaeota* accounted for <4% of the community. In particular, 3 OTUs were assigned to *M. thermautotrophicus*, *Methanoculleus thermophilus* and *Methanocorpusculum aggregans*. Interestingly, these microorganisms significantly increased between 15 and 120-folds ($p<0.05$), with *M. thermautotrophicus* being the most abundant methanogen (2.6% at the end of the experiment). Interestingly, BLASTn search against NCBI database revealed 100% identity with several microbial species, such as *Methanobacterium thermaggregans*, *M. wolfeii*, *M. thermophilus*, *M. thermoflexus*, and *M. defluvii*, in addition to *M. thermautotrophicus*, indicating that the most abundant methanogen populating the community was represented by a new species.

The presence of these hydrogenotrophs is in accordance with results shown in section 6.2, were these genera were dominant and increasing in upgrading reactors. The co-occurrence and dynamicity of different hydrogenotrophic methanogens, presumably performing the same function, rather than competition, is of particular interest because this could results in a more robust hydrogenotrophic methanogenic process, able to tolerate disturbances. Notably,

M. thermautotrophicus was found with the highest relative abundance in R2, the reactor presenting the best Y_{CH_4} and output-gas quality (96% CH_4 content). Additionally, R4, which performed at the best H_2 and CO_2 conversion efficiencies, showed the highest relative abundance of *M. thermophilus* (1.34%) suggesting a potential synergistic function between the two methanogens (Chapter 4).

In conclusion, the proliferation of hydrogenotrophic methanogens in concomitance with syntrophic bacteria demonstrates the selective effect of H_2 on community composition stimulating hydrogenotrophic methanogenic pathway.

7 Conclusions

Biological biogas upgrading is a promising way to extend biomethane utilization and reduce the dependence on fossil fuels, providing enhanced environmental and economic benefits of biogas technologies.

Results achieved in this PhD project demonstrate the feasibility of H_2-mediated biogas upgrading in both in-situ and ex-situ concepts.

Decoupling of biogas production and upgrading in a two-stage reactor provided efficient process performances, at both mesophilic and thermophilic conditions, with higher biomethanation and CO_2 conversion efficiency at thermophilic conditions. In this configuration, upon the H_2 addition, the CH_4 content increased up to 92% extending the potential uses of biomethane.

Moreover, H_2 transfer to the liquid phase represented an important limiting factor for H_2 availability for microorganisms. Therefore, this aspect was investigated in several reactor configurations for in-situ and ex-situ biogas upgrading. It was shown that the use of porous devices benefit the H_2 uptake as the gas-liquid contact area is increased and the gas retention time is extended. Moreover, the gas recirculation flow rate and the H_2 injection chamber design are fundamental elements that must be considered to maximize the gas retention time and thus the H_2 dissolution in the liquid media. Additionally, in upflow reactors for ex situ biogas upgrading, configurations containing larger pore size diffusion devices resulted in the best kinetics and output-gas quality, converting the total amount of H_2 and CO_2 injected to CH_4 and generating output gas with 96% CH_4 content that can be used as transportation fuel. This is explained by the higher mixing provided on reactor's liquid phase by larger pore size devices. In summary, from the application of these technologies, biogas upgrading can be achieved providing synergistic advantages for the overall renewable energy system and reducing greenhouse gases emissions.

Concerning the effect of H_2 on biogas microbiome, the innovative bionformatics approaches applied complemented each other providing a deeper insight into microbial community complexity. The application of *de novo* assembly followed by a binning strategy led to the most accurate description of the biogas microbial consortium identifying 236 genome bins. Moreover, a comparative study demonstrated the existence of a microbial group that could represent the core community of biogas production. This potential core community comprises resilient methanogenic archaea such as *Methanoculleus* and *Methanotermobacter* and bacteria of genus *Synthrophomonas* and phylum *Proteobacteria*.

Moreover, upon H_2 addition, decrease of aceticlastic methanogens and fermentative bacteria was observed. The concomitant increase of

hydrogenotrophic methanogens, such as *Methanothermobacter thermautotrophicus*, *Candidatus* Methanoculleus thermohydrogenotrophicum, and other *Methanoculleus* species, and syntrophic bacteria such as *Anaerobaculum mobile*, *Desulfuvibrio*, and some members of *Thermoanaerobacterales* and *Syntrophomonadaceae* confirms the selective force of the H_2 toward the hydrogenotrophic pathway enhancing the CO_2 consumption and thus the biogas upgrading.

8 Future Perspectives

In this PhD project, several novel reactors' configurations were designed to optimized in-situ and ex-situ biogas upgrading processes. Based on achieved results, the future perspective in terms of lab scale reactor configuration and process optimization is an innovative hybrid setup exploiting the findings of both in-situ and ex-situ biogas upgrading concepts. The proposed configuration consists of a double-stage reactor composed of a CSTR, working as a conventional anaerobic digester and where the H_2 is injected (in-situ biogas upgrading), and an up-flow reactor, receiving the upgraded biogas from the CSTR, together with the unutilized H_2, to be further upgraded (ex-situ biogas upgrading).

Moreover, in order to implement biogas upgrading technology in a commercial application, an economic and environmental impact assessment of the proposed process would be beneficial.

Similarly for full scale process applications, further investigations would be necessary:

- Due to the fact that H_2 assisted biogas upgrading technology is based on the surplus of electricity generated by solar or wind power, the system should be resilient to variable weather condition and thus to different input H_2 flow rates. Therefore, a study evaluating intermittent feeding of the hydrogenotrophic culture and required recovering time would be interesting to evaluate the feasibility of this process for an industrial application.
- Moreover, a detailed investigation of fluid dynamics and gas dispersion would be beneficial for the reproducibility of the process and to better understand the mechanisms of H_2 gas-liquid mass transfer.
- Finally, a preliminary study in pilot scale would be useful to evaluate the scalability of the technology and estimate process performances.

Finally, to achieve a deeper insight into the fundamentals of the biological biogas upgrading and acquire a clearer knowledge of the complexity of the microbial consortium populating biogas and biogas upgrading reactors, further investigations are needed. The intricate network of interactions among the microorganisms, resulting either in syntrophic relations or in competition, stresses the necessity for an analysis of the community going beyond the identification of the microbial species, but focused on their function in the biogas production process, providing the basis for future metatranscriptomic and metaproteomic studies.

9 References

Ahern, E.P., Deane, P., Persson, T., Ó Gallachóir, B., Murphy, J.D., 2015. A perspective on the potential role of renewable gas in a smart energy island system. Renew. Energy 78, 648–656.

Ahring, B.K., Westermann, P., Mah, R.A., 1991. Hydrogen inhibition of acetate metabolism and kinetics of hydrogen consumption by Methanosarcina thermophila TM-1. Arch. Microbiol. 157, 38–42.

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W., Nielsen, P.H., 2013. Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat. Biotechnol. 31, 533–8.

Albertsen, M., Karst, S.M., Ziegler, A.S., Kirkegaard, R.H., Nielsen, P.H., 2015. Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One 10, e0132783.

Alitalo, A., Niskanen, M., Aura, E., 2015. Biocatalytic methanation of hydrogen and carbon dioxide in a fixed bed bioreactor. Bioresour. Technol. 196, 600–605.

Angelidaki, I., Ellegaard, L., 2002. Anaerobic digestion in Denmark: Past, present and future, in: Anaerobic Digestion for Sustainability in Waste (Water) Treatment and Re-Use. Proceedings of 7th FAO/SREN-Workshop, 19-22 May 2002, Moscow, Russia. pp. 129–138.

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J.M., 2011. Biomethanation and its potential. Methods Enzymol. 494, 327–351.

Barampouti, E.M.P., Mai, S.T., Vlyssides, A.G., 2005. Dynamic modeling of biogas production in an UASB reactor for potato processing wastewater treatment. Chem. Eng. J. 106, 53–58.

Bassani, I., Kougias, P.G., Treu, L., Angelidaki, I., 2015. Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environ. Sci. Technol. 49, 12585–12593.

Bassani, I., Kougias, P.G., Angelidaki, I., 2016. In-situ biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresour. Technol. 221, 485–491.

Benjaminsson, G., Benjaminsson, J., Rudberg, R., 2013. Power-to-Gas – A technical review. SGC Rapp. 284, 67.

Bhattacharyya, D., Singh, K.S., 2010. Understanding the Mixing Pattern in an Anaerobic Expanded Granular Sludge Bed Reactor: Effect of Liquid Recirculation. J. Environ. Eng. 136, 576–584.

Boucias, D.G., Cai, Y., Sun, Y., Lietze, V.U., Sen, R., Raychoudhury, R., Scharf, M.E., 2013. The hindgut lumen prokaryotic microbiota of the termite Reticulitermes flavipes and its responses to dietary lignocellulose composition. Mol. Ecol. 22, 1836–1853.

Bryant, M.P., 1979. Microbial methane production—theoretical aspects. J. Anim. Sci. 48, 193–201.

Campanaro, S., Treu, L., Kougias, P.G., De Francisci, D., Valle, G., Angelidaki, I., 2016. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnol. Biofuels 9, 26.

Carton, J.G., Olabi, A.G., 2010. Wind/hydrogen hybrid systems: Opportunity for Ireland's wind resource to provide consistent sustainable energy supply. Energy 35, 4536–4544.

Chertkov, O., Sikorski, J., Brambilla, E., Lapidus, A., Copeland, A., Glavina Del Rio, T., Nolan, M., Lucas, S., Tice, H., Cheng, J.-F., Han, C., Detter, J.C., Bruce, D., Tapia, R., Goodwin, L., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Ovchinnikova, G., Pati, A., Chen, A., Palaniappan, K., Land, M., Hauser, L., Chang, Y.-J., Jeffries, C.D., Spring, S., Rohde, M., Göker, M., Bristow, J., Eisen, J. a, Markowitz, V., Hugenholtz, P., Kyrpides, N.C., Klenk, H.-P., 2010. Complete genome sequence of *Aminobacterium colombiense* type strain (ALA-1). Stand. Genomic Sci. 2, 280–289.

Cherubini, F., 2010. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energy Convers. Manag. 51, 1412–1421.

Cord-Ruwisch, R., Seitz, H.J., Conrad, R., 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch. Microbiol. 149, 350–357.

Cord-Ruwisch, R., Mercz, T.I., Hoh, C.Y., Strong, G.E., 1997. Dissolved hydrogen concentration as an on-line control parameter for the automated operation and optimization of anaerobic digesters. Biotechnol. Bioeng. 56, 626–634.

De Francisci, D., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2015. Microbial diversity and dynamicity of biogas reactors due to radical changes of feedstock composition. Bioresour. Technol. 176, 56–64.

Deng, L., Hägg, M.B., 2010. Techno-economic evaluation of biogas upgrading process using CO_2 facilitated transport membrane. Int. J. Greenh. Gas Control 4, 638–646.

Díaz, I., Pérez, C., Alfaro, N., Fdz-Polanco, F., 2015. A feasibility study on the bioconversion of CO_2 and H_2 to biomethane by gas sparging through polymeric membranes. Bioresour. Technol. 185, 246–53.

Drake, H.L., 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: past and current perspectives, in: Drake, H.L., Harold L. (Eds.), Acetogenesis. Springer US, Boston, pp. 3–60.

Energinet.dk. Annual report, 2014. . Frederica, Denmark. http://www.energinet.dk/SiteCollectionDocuments/Engelske%20dokumenter/Om%20os/Annual-report-2014.pdf

Energinet.dk. Electricity and Gas in Denmark, System Plan, 2015. Frederica, Denmark. https://www.energinet.dk/SiteCollectionDocuments/Engelske%20dokumenter/Om%20os/System%20Plan%202015.pdf

Garcia, J.L., Patel, B.K., Ollivier, B., 2000. Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. Anaerobe 6, 205–226.

Gomec, C.Y., 2010. High-rate anaerobic treatment of domestic wastewater at ambient operating temperatures: A review on benefits and drawbacks. J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. 45, 1169–1184.

Guiot, S.R., Cimpoia, R., Carayon, G., 2011. Potential of wastewater-treating anaerobic granules for biomethanation of synthesis gas. Environ. Sci. Technol. 45, 2006–2012.

Götz, M., Lefebvre, J., Mörs, F., McDaniel Koch, A., Graf, F., Bajohr, S., Reimert, R., Kolb, T., 2015. Renewable Power-to-Gas: A technological and economic review. Renew. Energy 85, 1371-1390.

Hahnke, S., Maus, I., Wibberg, D., Tomazetto, G., Pühler, A., Klocke, M., Schlüter, A., 2015. Complete genome sequence of the novel *Porphyromonadaceae* bacterium strain

ING2-E5B isolated from a mesophilic lab-scale biogas reactor. J. Biotechnol. 193, 34–6.

Hamelin, L., Wesnæs, M., Wenzel, H., Petersen, B.M., 2011. Environmental consequences of future biogas technologies based on separated slurry. Environ. Sci. Technol. 45, 5869–5877.

Hanreich, A., Schimpf, U., Zakrzewski, M., Schlüter, A., Benndorf, D., Heyer, R., Rapp, E., Pühler, A., Reichl, U., Klocke, M., 2013. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. Syst. Appl. Microbiol. 36, 330–338.

Hattori, S., 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23, 118–127.

Hess, M., Sczyrba, A., Egan, R., Kim, T.-W., Chokhawala, H., Schroth, G., Luo, S., Clark, D.S., Chen, F., Zhang, T., Mackie, R.I., Pennacchio, L.A., Tringe, S.G., Visel, A., Woyke, T., Wang, Z., Rubin, E.M., 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science 331, 463–7.

Holladay, J.D., Hu, J., King, D.L., Wang, Y., 2009. An overview of hydrogen production technologies. Catal. Today 139, 244–260.

Hung, C.H., Chang, Y.T., Chang, Y.J., 2011. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems - A review. Bioresour. Technol. 102, 8437–8444.

Iverson, V., Morris, R.M., Frazar, C.D., Berthiaume, C.T., Morales, R.L., Armbrust, E. V., 2012. Untangling Genomes from Metagenomes: Revealing an Uncultured Class of Marine Euryarchaeota. Science 335, 587–590.

Jiménez, D.J., Dini-Andreote, F., van Elsas, J.D., 2014. Metataxonomic profiling and prediction of functional behaviour of wheat straw degrading microbial consortia. Biotechnol. Biofuels 7, 92.

Jürgensen, L., Ehimen, E.A., Born, J., Holm-Nielsen, J.B., 2014. Utilization of surplus electricity from wind power for dynamic biogas upgrading: Northern Germany case study. Biomass and Bioenergy 66, 126–132.

Kleerebezem, R., Stams, A.J.M., 2000. Kinetics of syntrophic cultures: A theoretical treatise on butyrate fermentation. Biotechnol. Bioeng. 67, 529-543.

Kougias, P.G., Treu, L., Benavente, D.P., Boe, K., Campanaro, S., Angelidaki, I., 2016a. Ex-situ biogas upgrading and enhancement in different reactor systems. Bioresour. Technol. 225, 429-437.

Kougias, P.G., Treu, L., Campanaro, S., Zhu, X., Angelidaki, I., 2016b. Dynamic functional characterization and phylogenetic changes due to Long Chain Fatty Acids pulses in biogas reactors. Sci. Rep. 6, 28810.

Kougias, P.G., Campanaro, S., Treu, L., Zhu, X., Angelidaki, I., 2017. A novel archaeal species belonging to *Methanoculleus* genus identified via de-novo assembly and metagenomic binning process in biogas reactors. Anaerobe.

Krakat, N., Schmidt, S., Scherer, P., 2011. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. Bioresour. Technol. 102, 5692–5701.

Kramer, H.W., Bailey, J.E., 1991. Mass transfer characterization of an airlift probe for oxygenating and mixing cell suspensions in an NMR spectrometer. Biotechnol. Bioeng. 37, 205–209.

Kröber, M., Bekel, T., Diaz, N.N., Goesmann, A., Jaenicke, S., Krause, L., Miller, D., Runte, K.J., Viehöver, P., Pühler, A., Schlüter, A., 2009. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J. Biotechnol. 142, 38–49.

Laanbroek, H.J., Abee, T., Voogd, I.L., 1982. Alcohol Conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. Arch. Microbiol. 133, 178–184.

Lee, J.C., Kim, J.H., Chang, W.S., Pak, D., 2012. Biological conversion of CO_2 to CH_4 using hydrogenotrophic methanogen in a fixed bed reactor. J. Chem. Technol. Biotechnol. 87, 844–847.

Lee, J., Han, G., Shin, S.G., Koo, T., Cho, K., Kim, W., Hwang, S., 2016. Seasonal monitoring of bacteria and archaea in a full-scale thermophilic anaerobic digester treating food waste-recycling wastewater: Correlations between microbial community characteristics and process variables. Chem. Eng. J. 300, 291–299.

Levén, L., Eriksson, A.R.B., Schnürer, A., 2007. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. FEMS Microbiol. Ecol. 59, 683–693.

Li, Y.-F., Nelson, M.C., Chen, P.-H., Graf, J., Li, Y., Yu, Z., 2014a. Comparison of the microbial communities in solid-state anaerobic digestion (SS-AD) reactors operated at mesophilic and thermophilic temperatures. Appl. Microbiol. Biotechnol. 99, 969–980.

Li, J., Rui, J., Pei, Z., Sun, X., Zhang, S., Yan, Z., Wang, Y., Liu, X., Zheng, T., Li, X., 2014b. Straw- and slurry-associated prokaryotic communities differ during co-fermentation of straw and swine manure. Appl. Microbiol. Biotechnol. 98, 4771–4780.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Annals of the New York Academy of Sciences 1125, 171–189.

Lu, X., Zhen, G., Estrada, A.L., Chen, M., Ni, J., Hojo, T., Kubota, K., Li, Y.Y., 2015. Operation performance and granule characterization of upflow anaerobic sludge blanket (UASB) reactor treating wastewater with starch as the sole carbon source. Bioresour. Technol. 180, 264–273.

Luo, G., Angelidaki, I., 2012. Integrated biogas upgrading and hydrogen utilization in an anaerobic reactor containing enriched hydrogenotrophic methanogenic culture. Biotechnol. Bioeng. 109, 2729–2736.

Luo, G., Angelidaki, I., 2013a. Hollow fiber membrane based H_2 diffusion for efficient in situ biogas upgrading in an anaerobic reactor. Appl. Microbiol. Biotechnol. 97, 3739–44.

Luo, G., Angelidaki, I., 2013b. Co-digestion of manure and whey for in situ biogas upgrading by the addition of H_2: Process performance and microbial insights. Appl. Microbiol. Biotechnol. 97, 1373–1381.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., Angelidaki, I., 2012. Simultaneous hydrogen utilization and in situ biogas upgrading in an anaerobic reactor. Biotechnol. Bioeng. 109, 1088–1094.

Luo, G., De Francisci, D., Kougias, P.G., Laura, T., Zhu, X., Angelidaki, I., 2015. New steady-state microbial community compositions and process performances in biogas reactors induced by temperature disturbances. Biotechnol Biofuels 8, 3.

Martin, M.R., Fornero, J.J., Stark, R., Mets, L., Angenent, L.T., 2013. A single-culture bioprocess of *Methanothermobacter thermautotrophicus* to upgrade digester biogas by CO_2-to-CH_4 conversion with H_2. Archaea 2013, 1–11.

McHardy, A.C., García Martín, H., Tsirigos, A., Hugenholtz, P., Rigoutsos, I., 2007. Accurate phylogenetic classification of variable-length DNA fragments. Nat. Methods 4, 63–72.

McInerney, M. J., 1988. Anaerobic hydrolysis and fermentation of fats and proteins. in Zehnder A. J. B (Ed.), Biology of Anaerobic Microorganisms. John Wiley & Sons, New York, pp. 373–416.

McInerney, M.J., Bryant, M.P., 1981. Basic principles of bioconversions in anaerobic digestion and methanogenesis, in: Sofer, S.S., Zaborsky, O.R. (Eds.), Biomass Conversion Processes for Energy and Fuels. Springer US, Boston, pp. 277–296.

Merchuk, J.C., Contreras, A., García, F., Molina, E., 1998. Studies of mixing in a concentric tube airlift bioreactor with different spargers. Chem. Eng. Sci. 53, 709–719.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., Edwards, 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9, 386.

Mikkelsen, M., Jørgensen, M., Krebs, F.C., 2010. The teraton challenge. A review of fixation and transformation of carbon dioxide. Energy Environ. Sci. 3, 43–81.

Miller, C.S., Baker, B.J., Thomas, B.C., Singer, S.W., Banfield, J.F., 2011. EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. Genome Biol. 12, R44.

Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6, 441-454.

Narihiro, T., Kusada, H., Yoneda, Y., Tamaki, H., 2016. Draft Genome Sequences of *Methanoculleus horonobensis* Strain JCM 15517, *Methanoculleus thermophilus* Strain DSM 2373, and *Methanofollis ethanolicus* Strain JCM 15103, Hydrogenotrophic Methanogens Belonging to the Family *Methanomicrobiaceae*. Genome announcements, 4, e00199-16.

Ntougias, S., Bourtzis, K., Tsiamis, G., 2013. The microbiology of olive mill wastes. Biomed Res. Int. 784591.

Ohsumi, T., Nakashiki, N., Shitashima, K., Hirama, K., 1992. Density change of water due to dissolution of carbon dioxide and near-field behavior of CO_2 from a source on deep-sea floor. Energy Convers. Manag. 33, 685–690.

Oren, A., 2006. Life at High Salt Concentrations, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes: Volume 2: Ecophysiology and Biochemistry. Springer New York, New York, pp. 263–282.

Pauss, A., Andre, G., Perrier, M., Guiot, S.R., 1990. Liquid-to-Gas mass transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. Appl. Environ. Microbiol. 56, 1636–1644.

Pavlostathis, S.G., Giraldo-Gomez, E., 1991. Kinetics of anaerobic treatment: A critical review. Crit. Rev. Environ. Control 21, 411–490.

Pelletier, E., Kreimeyer, A., Bocs, S., Rouy, Z., Gyapay, G., Chouari, R., Rivière, D., Ganesan, A., Daegelen, P., Sghir, A., Cohen, G.N., Médigue, C., Weissenbach, J., Le Paslier, D., 2008. "*Candidatus* Cloacamonas acidaminovorans": Genome sequence

reconstruction provides a first glimpse of a new bacterial division. J. Bacteriol. 190, 2572–2579.

Petersson, A., Wellinger, A., 2009. Biogas upgrading technologies–developments and innovations, 2009. IEA Bioenergy 12–15. http://www.biogaspartner.de/fileadmin/biogas/Downloads/Studien/IEA-BiogasUpgradingTechnologies2009.pdf

Powar, M.M., Kore, V.S., Kore, S. V, Kulkarni, G.S., 2013. Review on Applications of UASB Technology for Wastewater Treatment 2, 125–133.

Ramsay, I.R., Pullammanappallil, P.C., 2001. Protein degradation during anaerobic wastewater treatment: Derivation of stoichiometry. Biodegradation 12, 247–257.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–14.

Sakai, S., Imachi, H., Hanada, S., Ohashi, A., Harada, H., Kamagata, Y., 2008. *Methanocella paludicola* gen. nov., sp. nov., a methane-producing archaeon, the first isolate of the lineage "Rice Cluster I", and proposal of the new archaeal order *Methanocellales* ord. nov. Int. J. Syst. Evol. Microbiol. 58, 929–936.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.

Schnürer, A., Zellner, G., Svensson, B.H., 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiol. Ecol. 29, 249-261.

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O., Huttenhower, C., 2012. Metagenomic microbial community profiling using unique clade-specific marker genes. Nat. Methods 9, 811–4.

Segata, N., Börnigen, D., Morgan, X.C., Huttenhower, C., 2013. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. Nat. Commun. 4, 2304.

Sevilla-Espinosa, S., Solórzano-Campo, M., Bello-Mendoza, R., 2010. Performance of staged and non-staged up-flow anaerobic sludge bed (USSB and UASB) reactors treating low strength complex wastewater. Biodegradation 21, 737–751.

Sharman, H., 2005. Why wind power works for Denmark. Civ. Eng. 158, 66–72.

Song, Y., Chen, B., Nishio, M., Akai, M., 2005. The study on density change of carbon dioxide seawater solution at high pressure and low temperature. Energy. 30, 2298–2307.

Sovacool, B.K., 2013. Energy policymaking in Denmark: Implications for global energy security and sustainability. Energy Policy 61, 829–839.

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7, 568–577.

Stolze, Y., Zakrzewski, M., Maus, I., Eikmeyer, F., Jaenicke, S., Rottmann, N., Siebner, C., Pühler, A., Schlüter, A., 2015. Comparative metagenomics of biogas-producing microbial communities from production-scale biogas plants operating under wet or dry fermentation conditions. Biotechnol. Biofuels 8, 14.

Su, Y., Li, B., Zhu, W.Y., 2013. Fecal microbiota of piglets prefer utilizing dl-lactate mixture as compared to d-lactate and l-lactate in vitro. Anaerobe 19, 27–33.

Suen, G., Weimer, P.J., Stevenson, D.M., Aylward, F.O., Boyum, J., Deneke, J., Drinkwater, C., Ivanova, N.N., Mikhailova, N., Chertkov, O., Goodwin, L. a, Currie,

C.R., Mead, D., Brumm, P.J., 2011. The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist. PLoS One 6, e18814.

Sun, Q., Li, H., Yan, J., Liu, L., Yu, Z., Yu, X., 2015. Selection of appropriate biogas upgrading technology-a review of biogas cleaning, upgrading and utilisation. Renew. Sustain. Energy Rev. 51, 521–532.

Tang, Y.Q., Ji, P., Hayashi, J., Koike, Y., Wu, X.L., Kida, K., 2011. Characteristic microbial community of a dry thermophilic methanogenic digester: Its long-term stability and change with feeding. Appl. Microbiol. Biotechnol. 91, 1447–1461.

Tatara, M., Makiuchi, T., Ueno, Y., Goto, M., Sode, K., 2008. Methanogenesis from acetate and propionate by thermophilic down-flow anaerobic packed-bed reactor. Bioresour. Technol. 99, 4786–4795.

Thrän, D., Billig, E., Persson, T., Svensson, M., Daniel-Gromke, J., Ponitka, J., Seiffert, M., Baldwin, J., Kranzl, L., Schipfer, F. and Matzenberger, J., 2014. Biomethane–status and factors affecting market development and trade, in Junginger, M., Baxter D. (Eds.), IEA Task, 40.

Tischer, K., Kleinsteuber, S., Schleinitz, K.M., Fetzer, I., Spott, O., Stange, F., Lohse, U., Franz, J., Neumann, F., Gerling, S., Schmidt, C., Hasselwander, E., Harms, H., Wendeberg, A., 2013. Microbial communities along biogeochemical gradients in a hydrocarbon-contaminated aquifer. Environ. Microbiol. 15, 2603–2615.

Tong, Z., McCarty, P., 1991. Microbial hydrolysis of lignocellulosic materials, in Isaacson, R. (Ed.), Methane from Community Wastes, Elsevier Applied Science, London, pp. 61–100.

Traversi, D., Villa, S., Lorenzi, E., Degan, R., Gilli, G., 2012. Application of a real-time qPCR method to measure the methanogen concentration during anaerobic digestion as an indicator of biogas production capacity. J. Environ. Manage. 111, 173–7.

Treu, L., Campanaro, S., Kougias, P.G., Zhu, X., Angelidaki, I., 2016a. Untangling the Effect of Fatty Acid Addition at Species Level Revealed Different Transcriptional Responses of the Biogas Microbial Community Members. Environ. Sci. Technol. 50, 6079–6090.

Treu, L., Kougias, P.G., Campanaro, S., Bassani, I., Angelidaki, I., 2016b. Deeper insight into the structure of the anaerobic digestion microbial community; The biogas microbiome database is expanded with 157 new genomes. Bioresour. Technol. 216, 260–266.

Turconi, R., Tonini, D., Nielsen, C.F.B., Simonsen, C.G., Astrup, T., 2014. Environmental impacts of future low-carbon electricity systems: Detailed life cycle assessment of a Danish case study. Appl. Energy 132, 66–73.

Turner, J., Sverdrup, G., Mann, M.K., Maness, P.C., Kroposki, B., Ghirardi, M., Evans, R.J., Blake, D., 2008. Renewable hydrogen production. Int. J. Energy Res. 32, 379–407.

Wallrabenstein, C., Schink, B., 1994. Evidence of reversed electron transport in syntrophic butyrate or benzoate oxidation by *Syntrophomonas wolfei* and *Syntrophus buswellii*. Arch. Microbiol. 162, 136–142.

Wang, C., Zuo, J., Chen, X., Xing, W., Xing, L., Li, P., Lu, X., Li, C., 2014. Microbial community structures in an integrated two-phase anaerobic bioreactor fed by fruit vegetable wastes and wheat straw. J. Environ. Sci. 26, 2484–2492.

Wasserfallen, A., Nölling, J., Pfister, P., Reeve, J., De Macario, E. C., 2000. Phylogenetic

analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. Int J Syst Evol Microbiol. 50, 43-53.

Weiland, P., 2010. Biogas production: current state and perspectives. Appl Microbiol Biotechnol 85, 849–860.

Werner, J.J., Knights, D., Garcia, M.L., Scalfone, N.B., Smith, S., Yarasheski, K., Cummings, T.A., Beers, A.R., Knight, R., Angenent, L.T., 2011. Bacterial community structures are unique and resilient in full-scale bioenergy systems. Proc. Natl. Acad. Sci. U. S. A. 108, 4158–63.

Westerholm, M., Roos, S., Schnürer, A., 2011. *Tepidanaerobacter acetatoxydans* sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from two ammonium-enriched mesophilic methanogenic processes. Syst. Appl. Microbiol. 34, 260–266.

Winter, J.U., Wolfe, R.S., 1980. Methane formation from fructose by syntrophic associations of *Acetobacterium woodii* and different strains of methanogens. Arch. Microbiol. 124, 73–79.

Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rákhely, G., Kovács, K.L., 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol. Biofuels 5, 41.

Xia, Y., Wang, Y., Fang, H.H.P., Jin, T., Zhong, H., Zhang, T., 2014. Thermophilic microbial cellulose decomposition and methanogenesis pathways recharacterized by metatranscriptomic and metagenomic analysis. Sci. Rep. 4, 6708.

Yang, S.J., Kataeva, I., Hamilton-Brehm, S.D., Engle, N.L., Tschaplinski, T.J., Doeppke, C., Davis, M., Westpheling, J., Adams, M.W.W., 2009. Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe "*Anaerocellum thermophilum*" DSM 6725. Appl. Environ. Microbiol. 75, 4762–4769.

Yue, Z., Chen, R., Yang, F., MacLellan, J., Marsh, T., Liu, Y., Liao, W., 2013. Effects of dairy manure and corn stover co-digestion on anaerobic microbes and corresponding digestion performance. Bioresour. Technol. 128, 65–71.

Zheng, M.X., Wang, K.J., Zuo, J.E., Yan, Z., Fang, H., Yu, J.W., 2012. Flow pattern analysis of a full-scale expanded granular sludge bed-type reactor under different organic loading rates. Bioresour. Technol. 107, 33–40.

Zinder, S.H., Koch, M., 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. 138, 263–272.

10 Papers

I Bassani, I., Kougias, P. G., Treu, L., Angelidaki, I. (2015). Biogas upgrading via hydrogenotrophic methanogenesis in two-stage Continuous Stirred Tank Reactors at mesophilic and thermophilic conditions. Environmental science & technology, 49(20), 12585-12593.

II Bassani, I., Kougias, P. G., Angelidaki, I. (2016). In-situ biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresource Technology, 221, 485-491.

III Bassani, I., Kougias, P. G., Treu, L., Porté, H., Campanaro, S., Angelidaki, I. (2017). Optimization of hydrogen dispersion in thermophilic up-flow reactors for ex-situ biogas upgrading. Bioresource Technology, 234, 310–319.

IV Treu, L., Kougias, P. G., Campanaro, S., Bassani, I., Angelidaki, I. (2016). Deeper insight into the structure of the anaerobic digestion microbial community; the biogas microbiome database is expanded with 157 new genomes. Bioresource technology, 216, 260-266.

V Treu, L., Campanaro, S., Kougias, P. G., Sartori, C., Bassani, I., Angelidaki, I. (2017). Genome-centric view of microcosms inhabiting thermophilic and mesophilic biogas upgrading reactors. Manuscript under preparation for submission to Biotechnology for biofuels.

In this online version of the thesis, **paper I-V** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

DTU Environment
Technical University of Denmark
Miljøvej, Building 113
2800 Kgs. Lyngby
Denmark
info@env.dtu.dk.

The Department of Environmental Engineering (DTU Environment) conducts science based engineering research within six sections: Water Resources Engineering, Water Technology, Urban Water Systems, Residual Resource Engineering, Environmental Chemistry and Atmospheric Environment.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

Department of Environmental Engineering
Technical University of Denmark

DTU Environment
Bygningstorvet, building 115
2800 Kgs. Lyngby
Tlf. +45 4525 1600
Fax +45 4593 2850

www.env.dtu.dk